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BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENTIATION DURING MORPHOGENESIS

XIV. THE NUCLEIC ACIDS OF THE DEVELOPING CEREBRAL CORTEX AND LIVER OF THE FETAL GUINEA PIG [1]

JOSEFA B. FLEXNER AND LOUIS B. FLEXNER [2]
Department of Embryology, Carnegie Institution of Washington, Baltimore

THREE FIGURES

The observations reported here were undertaken to test by chemical methods the relation between the concentration of pentose nucleic acid (PNA) and the rate of protein synthesis in fetal nerve cells and hepatic cells. There is now a great deal of evidence that rapidly growing cells and others that secrete large amounts of protein have an increased concentration of PNA in their cytoplasm (Brachet, '50; Caspersson, '50); this evidence has led to the view that PNA is intimately concerned in some unknown way with protein synthesis. Hyden ('47), using Caspersson's technique of ultraviolet microspectrophotometry, has reported that young anterior horn cells during the whole period of their embryonic development contain cytoplasmic nucleotides in high concentration in correspondence with their rapid rate of protein synthesis. Bodian ('47) points out that the regenerating neurone starts to reconstitute its axon with little PNA in the perikaryon but that increasing quantities appear with more active synthesis of axoplasm. Amounts of PNA in excess of that in the adult have been found in fetal (Caspersson, '50) and in

[1] This investigation was supported by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council and by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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regenerating liver (Stowell, '48; Novikoff and Potter, '48; Drabkin, '47).

We now have considerable information about the nerve cells of the frontal cerebral cortex and of the hepatic cells of the fetal guinea pig with which to estimate the synthetic activity of these cells at various stages of development. In the case of the nerve cells, active synthesis revealed by the rapid elaboration of processes (Peters and Flexner, '50) apparently with a protein concentration equal to that of the cell bodies from which they develop (Flexner and Flexner, '50), begins at the 41st to 45th days of gestation (term, 66 days). Is the onset of intense synthetic activity to be correlated with an increase in overall concentration of PNA in the cytoplasm of the body of the nerve cell, the perikaryon? In the case of the liver, the increase in the weight of the organ and of its protein has been established for a considerable part of gestation, so that the relative growth rate can be calculated at any time during this period (Flexner and Flexner, '50). Is the apparent rate of protein synthesis proportional to the concentration of PNA as has been demonstrated for bacteria (Malmgren and Heden, '47)?

Knowledge of the concentration of desoxypentose nucleic acid (DNA) has become particularly revealing since the demonstration (Boivin, Vendrely and Vendrely, '48; Vendrely and Vendrely, '49; Mirsky and Ris, '49) that this substance may be present in quite constant amounts in the nuclei of mature somatic cells. On this basis the ratio of the number of cells in different samples of tissue can be obtained from the relative amounts of DNA contained in the tissues; and indeed when the absolute amount of DNA per nucleus is known, as is now the case for several animals, the number of cells per unit weight of tissue can probably be estimated with considerable accuracy. This approach has great potential value in analyzing many aspects of growth which may be accompanied by large changes in the number of cells per unit volume of tissue. In the absence of direct evidence, we have supposed that the nuclei of the cells of the cerebral

cortex, irrespective of their age, contain a constant amount of DNA and that this same assumption is without serious error for our purposes when applied to the liver of the fetal guinea pig (Swift, '50).

METHODS

Cerebral cortex was obtained as far as possible from the frontal cortex only, areas f and f′ of Fortuyn ('14). To minimize variations, samples of liver were preferably taken

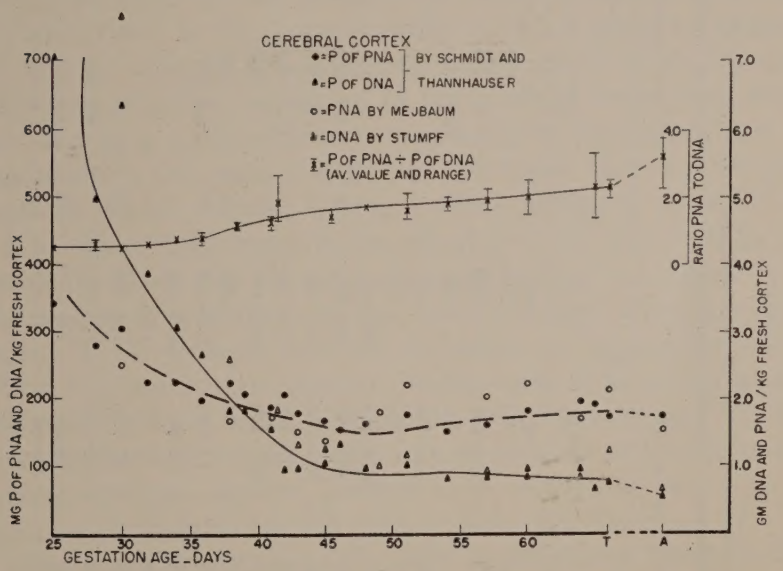

Fig. 1 Changes with developmental age of the amounts of PNA and DNA and of the ratio PNA to DNA per kilogram fresh, frontal cerebral cortex. T = term; A = adult. The curves are fitted to the data obtained by the method of Schmidt and Thannhauser.

from the tip of the same lobe of each animal. RNA and DNA were first determined by the method of Schmidt and Thannhauser ('45). As a check, nucleic acids were extracted from additional samples of tissue using Schneider's procedure ('45); RNA in the extract was determined by Mejbaum's method ('39) and DNA by the method of Stumpf ('47).

TABLE 1

Range of values from which the average values of the graphs were calculated. Phosphorus (P) of DNA and of PNA by the method of Schmidt and Thannhauser; "check estimations" by the methods of Stumpf and Mejbaum

GEST. AGE	NO. EXP.	*P of DNA and PNA per kg wet wt. of:* LIVER DNA	LIVER PNA	CORTEX DNA	CORTEX PNA	NO. EXP.	*Check estimations per kg wet wt. of:* LIVER DNA	LIVER PNA	CORTEX DNA	CORTEX PNA
days		*mg*	*mg*	*mg*	*mg*		*gm*	*gm*	*gm*	*gm*
25	1	471	776	703	340					
28	2	696	773	474–494	279	1	7.0	4.4		
30	1			637	308	2	10.9–13.1	3.0–7.6	7.5–7.9	2.3–2.7
32	1	838	831	384	222					
34	1	835	782	305	223					
36	2	674–892	621–676	219–315	190–205					
38–39	3	880–1030	660–771	180–186	202–224	5	13.2–19.2	4.0–5.1	2.4–2.8	1.6–1.7
41	4	810–900	629–750	123–175	162–202	2	10.1–10.3	7.5–8.5	1.8–1.9	1.3–2.2
42–43	5	282–945	553–672	69–138	174–203	4	15.2–20.4	5.7–9.1	1.3–1.5	1.0–2.0
45–46	2	715	617	102–132	152–164	1	12.9	8.9	1.3	1.3
48	2	820–860	605–702	96	161	3	9.0	7.7	0.8–1.3	1.6–2.4
51	4	483–1017	317–710	71–125	150–194	3	8.9–9.5	5.1–10.4	1.1–1.2	2.0–2.4
54	2	725–987	587–665	74–85	138–152					
57	2	529–620	400–449	67–107	148–171	1			0.9	2.1
60	2	175–577	402–515	63–133	158–206	1			0.9	2.2
64–65	5	448–915	368–573	58–129	175–214	2	8.7–9.1	5.2–7.0	0.8–1.0	1.7–1.8
66	4	106–226	215–350	69–85	133–210	3	1.6–3.3	2.5–2.7	1.2–1.3	1.9–2.5
Adult	9	117–464	474–713	36–84	91–229	12	2.0–3.6	2.1–10.9	0.3–1.0	0.8–2.9

RESULTS

Cerebral cortex. The ranges of values for PNA and DNA per kilogram wet weight of cortex at various gestation ages are given in table 1; average values and the curve fitted to them, in figure 1. It is a characteristic of the findings that considerable variation in the concentrations of both nucleic acids may be observed at a particular stage of development; this is most pronounced in the adult. The variation in DNA is probably a reflection of differences in the density of the total cell population in the samples of tissue taken for analysis. Irregularities in the concentration of PNA may be because of one of several circumstances: differences in the density of cell population, variation in the average amount of PNA present in a particular cell type such as the nerve cell or variation in the ratio of different cell types (nerve cells, glia, cells of blood vessels) which contain different amounts of PNA. That differences in the density of the total cell population is likely not an adequate explanation is evident from the range of variation of the ratio PNA to DNA given in figure 1. It follows that the variation in concentration of PNA at a particular stage of development is probably attributable in part at least to fluctuations in the average amount present per cell and that this may be because of irregularities in the average amount present in a particular cell type or because of variation in the ratio of different cell types.

The variations which have been encountered, however, are not large enough to obscure the trend of the data as shown in figure 1. The average amount of DNA per unit weight of cortex at the 25th day of gestation is 7 times or more that at term or in the adult, the decline becoming asymptotic at about the 45th day. This conclusion is based upon both the results obtained with the Schmidt and Thannhauser method and the check determinations made with that of Stumpf. The curve agrees substantially with that found for the change with gestation age in number of nerve cell nuclei per unit volume of cortex (Peters and Flexner, '50) and is to be considered a reflection of the rapid increase in spacing among cells during

the early maturation of the cortex. The average concentration of PNA as determined by the method of Schmidt and Thannhauser at the 25th day is more than twice that at the minimum level observed at about the 46th day; after the 46th day there appears to be a gradual and relatively slight increase to term. The same general change is evident from the results obtained with Mejbaum's method; a decline to a minimum value at about the 45th day followed by a subsequent increase in concentration to term, the latter being greater than that observed with the Schmidt and Thannhauser method.

It now becomes necessary to analyze the observations in terms of the primary problems in which we are interested. The volume of the cytoplasm of the average nerve cell body, the perikaryon, has been found (Peters and Flexner, '50) to increase linearly from the 30th day of gestation to term. Evidence of greatly increased synthetic activity of the nerve cells is first observed from the 41st to 45th day of gestation when processes first appear and then grow rapidly and there is a consequent diminution in the volume of the extracellular phase (Flexner and Flexner, '49). With the assumption already stated that the nuclei of the cells of the cortex contain a constant amount of DNA, the data permit an answer to the question: Is the onset of this increased synthetic activity correlated with an increase in the average amount of PNA per cell? As shown in figure 1 the ratio of PNA to DNA first begins to increase at about the 39th day, the increase continuing to term and the adult so that at term the average amount of PNA per cell is about 4 times that observed at the 36th day and before. This result obtained with the Schmidt and Thannhauser method is in agreement with that given by the methods of Stumpf and Mejbaum. It is reasonable to suppose, on the basis of an increase in the amount of basophilic substances (Peters and Flexner, '50), that the nerve cells share to at least a considerable degree in this increase and that it is not entirely because of an increase in the average amount of PNA present in glia and other cells. To test the thesis in which we are principally interested, however, it is

necessary to gain a measure of the change in concentration of PNA in the perikaryon itself. This cannot be accomplished directly because we do not know the amount of PNA present in glia and other cells and in the nerve processes. We can assume, however, in agreement with their relative basophilia that the amount of PNA in cells other than nerve cells is slight and suppose that all the cytoplasmic PNA belongs to the perikaryon. The amount of perikaryon per unit weight of frontal cortex has previously been estimated (Peters and Flexner, '50). In these estimations the perikaryon was con-

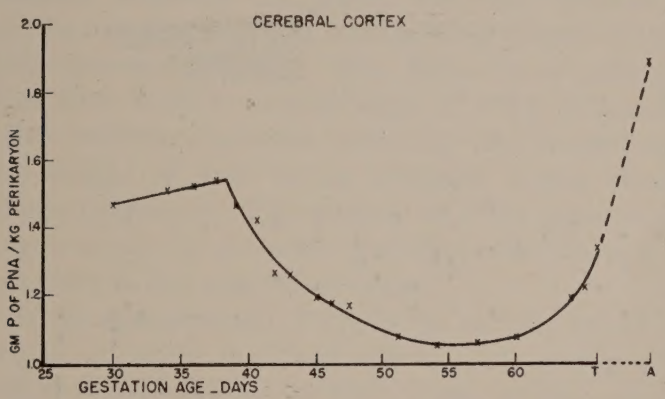

Fig. 2 Change with developmental age of the amount of P of PNA per kilogram cytoplasm of nerve cell body. Data derived as described in the text.

sidered to extend to the base of the processes, usually evidenced by a change in the contour of the cell wall. When the published values for the submolecular cortex are corrected at early stages of gestation to total cortex, an estimate of the overall concentraion of PNA per unit weight of perikaryon is obtained by dividing the average amount of PNA per unit weight of cortex by the amount of cytoplasm of nerve cell body in that weight of cortex. As is shown in figure 2, when the data are treated in this way, it appears that the overall concentration of PNA in the perikaryon begins quite sharply to decrease just before the critical period of 41–45 days and two weeks later reaches a minimum value approxi-

mately one-third lower. An apparent decrease in concentration of PNA during the critical period is also observed when the more limited data obtained with Mejbaum's method are subjected to the same treatment; on the basis of these findings, however, it appears that at the 51st day the concentration of PNA is restored to the level observed in the precritical stage (with Mejbaum's method, the amount of PNA in grams per kilogram perikaryon is 13 at the 30th and 38th days; 14, 41st day; 11, 43rd day; 9, 45th day; 10, 48th day;

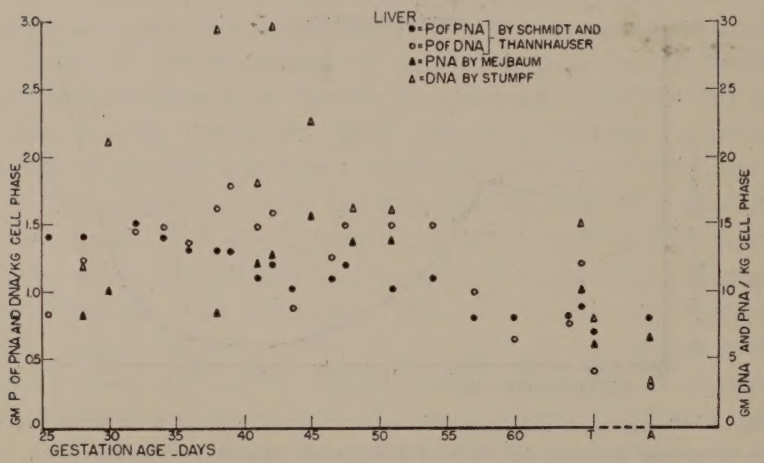

Fig. 3 Change with developmental age of the amounts of PNA and DNA per kilogram hepatic cell phase, free of fat and glycogen.

13, 51st day and to term). On the basis of this treatment of the data it follows that there is no evidence for an increase in overall concentration of PNA in the perikaryon of the nerve cell during the period of increased synthetic activity associated with rapid elaboration of processes. Indeed the evidence indicates that during the beginning of the synthetic process at least there is a decline in the overall concentration of PNA although, as will later be discussed, the appearance of Nissl bodies is evidence for an increase in concentration of PNA in restricted portions of the cytoplasm at this time.

Liver. The ranges of values per kilogram wet weight for PNA and DNA at various gestation ages are given in table 1; average values per kilogram cell phase free of fat and glycogen, in figure 3. As in the cerebral cortex, it is a characteristic of the findings that considerable variations in the concentration of both nucleic acids may be observed at a particular stage of development. In the liver this is most pronounced for DNA. The relatively high and irregular values for DNA at intermediate stages of gestation are probably related to the presence of large and variable numbers of erythroblasts evidence for which was also obtained in a study of the extracellular and intracellular phases of the fetal liver (Flexner and Flexner, '49). Since these cells are reported to have a high concentration of PNA (Thorell, '47) they will in addition increase values for the amount of PNA per unit weight of liver to an irregular degree depending upon their abundance. The amount of PNA present in mature erythrocytes is so small that they contribute a negligible quantity to the analytical values.

How is the concentration PNA in the cellular phase related to the rate at which the liver cell is synthesizing protein? The average concentration of PNA in the cellular phase free of fat and glycogen is given in figure 3. If these values were expressed in terms of the cytoplasm of the cell phase rather than the total cell phase the indicated concentration would be increased between 20 and 25% since Peters ('51) using Chalkley's ('43) method has found that the ratio of nuclear to cytoplasmic volume of the hepatic cells fluctuates between 0.2 and 0.26 from the 28th to about the 60th day of gestation. Thereafter the ratio diminishes coincidently with and because of the deposition of large amounts of fat and smaller amounts of glycogen in the cytoplasm. Use of the total cellular phase free of fat and glycogen, rather than cytoplasm alone can therefore be considered to have no effect on the relative values throughout the period of gestation which has been studied. The Schmidt and Thannhauser method

shows a consistent decline in the concentration of PNA from about the 30th day of gestation to term, the value at term being indistinguishable from that of the adult. Over this period the concentration of PNA drops to about 50% of its earliest value. More limited estimations made with Mejbaum's method, which is probably less reliable for reasons such as those mentioned by Schmidt and Thannhauser ('45), show even less difference between the earliest stages and term though the maximum range of the average values at different stages of development is much like that found with the Schmidt and Thannhauser method. The actual concentration of PNA in the cell phase at early and intermediate stages of development is less by an unknown factor than indicated in the graph. This is because the PNA present in extravascular erythroblasts at these stages is ascribed to the hepatic cells. The concentration of PNA in the cell phase from the 30th day of gestation to term consequently diminishes by less than a factor of two. Cellular protein, however, shows a much greater variation in its rate of synthesis over this same period. From the 30th day to term the relative percentage growth rate per day of the hepatic cells decreases from 25 to three (Flexner and Flexner, '50), i.e., the rate at which the hepatic cells reproduce their own weight, declines by a factor of 8 during this period. (The relative percentage growth rate is defined as $dW/Wdt \times 100$ where $W =$ weight of the liver at a particular time and $dW/Wdt =$ rate of gain in weight at this time.) Since the protein of the hepatic cells is essentially constant in concentration over this period (Flexner and Flexner, '50), change in relative growth rate of the hepatic cells is directly proportional to change in the relative rate at which protein is being synthesized; the rate of protein synthesis per unit weight of hepatic cells therefore declines by a factor of 8 during the last half of gestation. It must be concluded, consequently, that the relatively high level of protein synthesis observed in the liver at 30 days is not matched by a correspondingly high

concentration of PNA. It is also to be noted that the concentration of PNA at term when the liver is still growing, though slowly in terms of its relative growth rate, is indistinguishable from that of the adult.

Because of the large and variable number of extravascular erythroblasts, the ratio of PNA to DNA is without clear meaning. Using the Schmidt and Thannhauser data, the ratio fluctuates around a mean value of 0.9 to the 55th day of gestation; then increases to two at later prenatal stages.

DISCUSSION

The treatment which we have made of our findings on the cerebral cortex gives a falsely high value for the concentration of PNA in the cytoplasm of the nerve cell body because all of the PNA of the cortex is considered to belong to the perikaryon. It is possible that the apparent concentration of PNA in the perikaryon may be raised in this way to a greater degree at one stage of development than another. Thus if the PNA derived from cells other than nerve cells is greater in the precritical stage (before 41 days) than in the postcritical stage (after 45 days), the relative apparent concentration of PNA in the perkaryon of the precritical stage will be too high. The excellent agreement from the 34th day of gestation to term between the curve for the number of nuclei per unit volume of cortex (Peters and Flexner, '50) and concentration of DNA in the cortex is evidence against this assumption and in addition the relatively slight basophilia of cells other than nerve cells suggest that they contribute a relatively slight amount of PNA to the total. Processes of nerve cells are essentially absent up to the critical period of 41 to 45 days so that whatever PNA is present in them will in our treatment raise the apparent concentration of PNA in the perikaryon only in the postcritical stage. In spite of the addition of whatever PNA is derived from nerve cell processes there is no evidence of an increase in overall concentration of PNA in the perikaryon at the time of increased synthetic activity associated with the

rapid elaboration of processes; indeed the evidence indicates that there is a decrease in overall concentration of PNA at about this time with subsequent recovery.

This does not mean that the amount of PNA per perikaryon does not increase at the critical period. The increase in the ratio of PNA to DNA indicates that on the average the amount of PNA per perikaryon does increase at this time and that the perikaryon continues to gain PNA to term and in postnatal life. But the volume of the perikaryon (Peters and Flexner, '50) increases more rapidly from the 38th to the 55th day than does its quantity of PNA so that the average concentration of PNA decreases. Increase in the ratio of PNA to DNA is correlated with the appearance of the densely basophilic Nissl bodies, evidence that local concentration of PNA within the cytoplasm is increased while its overall concentration in the perikaryon is declining.

It must be emphasized that our approach yields an average value for the concentration of PNA per unit weight of perikaryon and consequently gives no information on the large irregularities from cell to cell which are indicated by variations in their basophilia (Peters and Flexner, '50). Histochemical techniques utilizing microspectrophotometry appear to be ideally suited to measurement of this variation in concentration of PNA but not readily adapted to the problem of measuring the average overall concentration of PNA. The large number of observations required because of the variation in basophilia and hence presumably in concentration of PNA from cell to cell and indeed from one part of the perikaryon to another appear to make the method difficult of application in the fetal cerebral cortex.

In the liver, increase in the apparent rate of synthesis of protein per unit weight of cytoplasm at early stages of gestation is correlated with an apparent elevation in the concentration of PNA. This increase in concentration of PNA, however, is not proportional to the increase in synthetic activity; whereas the fetal cell at the 30th day of gestation is adding to its protein at a rate 8 times that at term, the

concentration of PNA is something less than twice that at term. It will be recognized that in using observations on the gain in the amount of protein we are using only the most obvious indication of synthetic activity relative to protein. We do not know the rate of degradation of the protoplasm of the hepatic cells during fetal growth and hence the rate of total rather than of net synthesis. Nor do we know the rate of synthesis of other proteins, those of the red cells when the liver is an active hematopoietic organ and those of the plasma.

In view of these findings on the cerebral cortex and the liver and that of Herrmann and Nicholas ('49), who found that increased rate of protein synthesis by fetal rat muscle is correlated with a decrease in concentration of PNA, it appears necessary to modify the generalization that increased rate of protein synthesis is accompanied by increased overall concentration of PNA in the cytoplasm of the cell. It does seem generally true that substantial differences in rate of protein synthesis are accompanied by a change in the concentration of PNA. In the great majority of instances this change is in the positive direction. But as evidenced by fetal rat muscle and cerebral cortex of the fetal guinea pig the change may be in the negative direction; i.e., increased synthetic activity may be correlated with decrease in overall concentration of PNA. Moreover, as shown by our results on liver the change in concentration of PNA may not be proportional to the change in rate of protein synthesis. As suggested by the views of Brachet ('50) it may be more important to consider the concentration of PNA in the "granules" of the cytoplasm rather than its overall concentration. If this be so, the nerve cell as evidenced by the appearance of Nissl bodies, gains in concentration of PNA in certain portions of the cytoplasm as its processes appear and grow, and satisfies the thesis that increased rate of protein synthesis is accompanied by increase in concentration of PNA within restricted parts of the cell.

We are obliged to Mrs. Joseph E. Thomas for technical assistance.

SUMMARY

Evidence of greatly increased synthetic activity of the nerve cells of the frontal cerebral cortex of the fetal guinea pig is observed from the 41st to 45th days of gestation when processes first appear and then grow rapidly. Using chemical methods, it appears that this increase in synthetic activity is correlated with an increase in the average amount of PNA per cell. The average concentration of PNA in the perikaryon appears to decrease, however, during and for some time after the period when processes appear since the volume of the perikaryon increases more rapidly than does its content of PNA.

In the liver, increase in the apparent rate of synthesis of protein per unit weight of cytoplasm at early stages of gestation is correlated with an apparent elevation in the concentration of PNA. This increase in concentration of PNA is not proportional to the increase in synthetic activity; whereas the fetal cell at the 30th day of gestation is adding to its protein at a rate 8 times that at term, the concentration of PNA at the 30th day is something less than twice that at term.

These findings on the cerebral cortex and the liver are discussed in the light of current concepts of the relationship between concentration of PNA and rate of protein synthesis.

REFERENCES

Bodian, D. 1947 Nucleic acid in nerve cell regeneration. Symp. Soc. Exp. Biol., *1:* 163–178. University Press, Cambridge.

Boivin, A., R. Vendrely and C. Vendrely 1948 L'acide desoxyribonucleique du noyau cellulaire. Compt. Rend d. Seances d. Acad. d. Sci., *226:* 1061–1063.

Brachet, J. 1950 The localization and the role of ribonucleic acid in the cell. Ann. N. Y. Acad. Sci., *50*, Art 8: 861–869.

Caspersson, T. O. 1950 Cell growth and cell function. First edition. W. W. Norton & Co., N. Y.

Chalkley, H. W. 1943 Method for the quantitative morphologic analysis of tissues. J. Nat. Cancer Inst., *4:* 47–53.

DRABKIN, D. L. 1947 Liver regeneration and cytochrome C metabolism. J. Biol. Chem., *171:* 395–408.

FLEXNER, J. B., AND L. B. FLEXNER 1949 Biochemical and physiological differentiation during morphogenesis. IX. The extracellular and intracellular phases of the liver and cerebral cortex of the fetal guinea pig as estimated from distribution of chloride and radiosodium. J. Cell. and Comp. Physiol., *34:* 115–127.

———— 1950 Biochemical and physiological differentiation during morphogenesis. XI. The effect of growth on the amount and distribution of water, protein and fat in the liver and cerebral cortex of the fetal guinea pig. Anat. Rec., *106:* 413–427.

FORTUYN, A. B. D. 1914 Cortical cell-lamination of the hemispheres of some rodents. Arch. Neurol. and Psychiat., *6:* 221–354.

HERRMANN, H., AND J. S. NICHOLAS 1949 Nucleic acid content of whole homogenates and of fractions of developing rat muscle. J. Exp. Zool., *112:* 341–359.

HYDEN, H. 1947 Protein and nucleotide metabolism in the nerve cell under different functional conditions. Symp. Soc. Exp. Biol., *1:* 152–162. University Press, Cambridge.

MALMGREN, B., AND C. G. HEDEN 1947 Quoted by Caspersson, T. O., in Cell Growth and Cell Function. First edition, 1950. W. W. Norton & Co., N. Y.

MEJBAUM, W. 1939 Estimation of small amounts of pentose especially in derivatives of adenylic acid. Z. Physiol. Chem., *258:* 117–120.

MIRSKY, A. E., AND H. RIS 1949 Variable and constant components of chromosomes. Nature, *163:* 666–667.

NOVIKOFF, A. B., AND V. R. POTTER 1948 Biochemical studies on regenerating liver. J. Biol. Chem., *173:* 223–238.

PETERS, V. B. 1951 Unpublished observations.

PETERS, V. B., AND L. B. FLEXNER 1950 Biochemical and physiological differentiation during morphogenesis. VIII. Quantitative morphologic studies on the developing cerebral cortex of the fetal guinea pig. Am. J. Anat., *86:* 133–161.

SCHMIDT, G., AND S. J. THANNHAUSER 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem., *161:* 83–89.

SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293–303.

STOWELL, R. E. 1948 Nucleic acids and cytologic changes in regenerating rat liver. Arch. Path., *46:* 164–178.

STUMPF, P. K. 1947 A colorimetric method for the determination of desoxyribonucleic acid. J. Biol. Chem., *169:* 367–371.

SWIFT, H. H. 1950 Desoxyribose nucleic acid content of animal nuclei. Physiol. Zool., *23:* 169–198.

THORELL, B. 1947 The relation of nucleic acids to the formation and differentiation of cellular proteins. Symp. Quant. Biol., *12:* 247–255. Biol. Lab. Cold Spring Harbor.

VENDRELY, R., AND C. VENDRELY 1949 La teneur du noyau cellulaire en acid desoxyribonucleique a travers les organes, les individus et les especes animales. Experientia, *5:* 327–329.

ELECTRICAL PHENOMENA IN NERVE

III. FROG SCIATIC NERVE [1]

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SIX FIGURES

INTRODUCTION

Compounds which block nerve activity may operate (a) by depolarizing the fibers — for example, by metabolic inhibition — thereby limiting the energy available for conduction, or (b) by a less clearly defined mechanism which leaves the polarization unaltered or enhanced (e.g., Bishop, '32). The latter type have been referred to as "stabilizers" (Bennett and Chinburg, '46). Conversely, other substances cause repetitive firing following a single stimulus, and frequently spontaneous activity (e.g., Welsh and Gordon, '47); these will be referred to as "unstabilizers."

It has been the purpose of this research to determine in detail the generality of certain new relationships in frog nerve between stabilization and the resting potential which suggest that membrane permeability to potassium is involved (Shanes, '48a), and which may therefore serve as a broad basis for testing directly for the role of potassium movement. To this end several stabilizers (e.g., pyribenzamine,[3] cocaine, procaine, and yohimbine) and unstabilizers (e.g., veratrine and

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[3] Very kindly provided by Dr. F. F. Yonkmann of Ciba Pharmaceutical Products, Inc.

DDT) have been examined chiefly with respect to their effects on the following: (a) The level of polarization, (b) the depolarization by anoxia, and (c) the elevaton of the polarized state by 5% CO_2 in O_2. Observations on the threshold and action potential will also be described.

METHOD

Procedures previously described (Shanes, '48b, '49a) were employed. Three paired nerves from R. pipiens were mounted at one time in a lucite chamber through which humidified oxygen flowed. In most experiments the potential difference between two intact portions of each nerve was recorded. This will be referred to as the demarcation potential, and the changes at one of these regions will be assumed to be proportional to changes in the "resting potential." The nerves passed through vaseline sealed slots in a partition which divided the chamber into two compartments; this permitted the distal half of each nerve to remain as a reference in oxygen while the other half could be subjected to 5% CO_2 in oxygen or to Linde highly purified tank nitrogen (99.9%). The central region of each nerve was either placed into, or mounted on a filter paper strip inserted into, one end of a U-tube containing Ringer's or other experimental solution. The filter paper was usually used in gas exchange experiments, and was preceded by a two-hour soaking of the entire nerve in oxygenated control and experimental solutions. The U-tube was employed directly when the effects of a localized change in solution, accomplished from outside the chamber by way of the U-tube, were of interest. The central region, in conjunction with a Bishop bridge arrangement, usually served for stimulation and observaton of the action potential of individual nerves; a silver wire, in individual pools of Ringer's in contact with the proximal ends of the nerves, provided the anode. Demarcation and action potentials were both recorded at the central region relative to the more distal area. When monophasic recording was desired, the distal ends were crushed and introduced into isotonic KCl; a drop of KCl

solution was also applied to the nerves 0.5 cm from the trough to hasten the development of monophasicity. Instrumentation and other mounting details are described in the cited references.

The Ringer's employed contained the usual electrolytes in the following concentrations: 107 mM NaCl, 1.7 mM KCl, 1.1 mM $CaCl_2$, and a pH 7.4 all-sodium Sørensen phosphate buffer equivalent to 1 mM NaCl. Phosphate buffer, the only experimental agent added in osmotically significant amounts, replaced an osmotic equivalent of NaCl. Additions involving a change of only a few per cent in tonicity, which have negligible effects (Shanes, '48b), were made without altering the other concentrations. All solutions were adjusted to a pH of 7.3–7.4. Experiments were carried out at room temperature (20–25°C.).

The hydrochlorides of the organic agents were used exclusively. In the majority of the experiments these were allowed to act three to 5 hours prior to the introduction of such variables as CO_2 and N_2; after the two-hour preliminary soaking of the whole nerves, the preparations were equilibrated for the remainder of the time in the chamber until sufficiently stable baselines were obtained. The concentrations employed were sufficient to assure 100% consistency of results as determined by a comparison of paired nerves subjected to CO_2 and N_2; this is the case for all data given in the tables. Such consistency of the data, typical of the technique of pairing (Shanes, '48b), eliminated the need for statistical procedures; consequently the variability is expressed only where absolute values rather than the direction of the change are of special interest.

The polarization changes directly induced by application of stabilizers, on the other hand, were frequently so slow or small that baseline drift, which gradually changes its time course, rendered uncertain the time of maximal increase and the exact magnitude of the increment. Controls from the opposite side of the same animal, run simultaneously in the same chamber and subjected to a change in Ringer's at the

same time, served to rule out extraneous factors and permitted a decision concerning the reality and approximate magnitude of the changes observed. Threshold and spike height were similarly controlled, but experimental variations in these parameters, when they occurred, were usually more readily detected. Threshold was measured as the shock (condenser discharge) height, seen on the cathode ray oscilloscope, required to evoke a response detectable at high amplification.

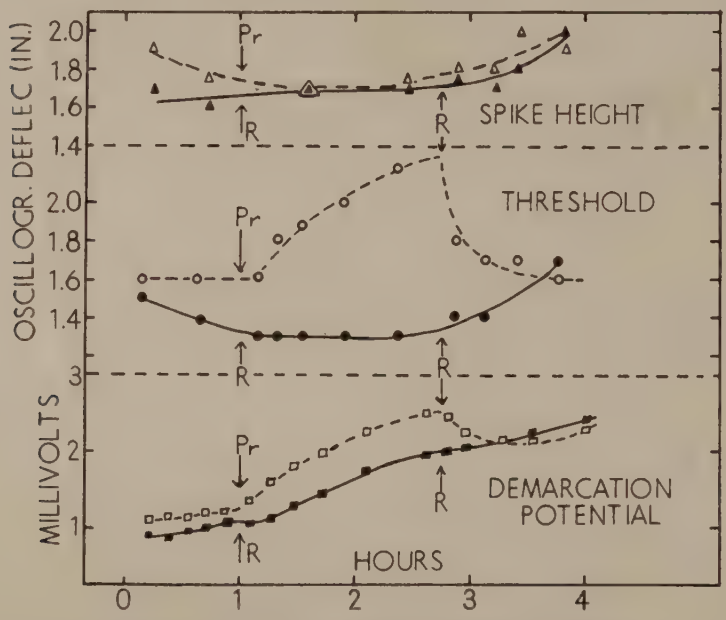

Fig. 1 The response of a nerve to 20 mg % procaine Ringer (Pr) and to its replacement with Ringer (R) with respect to demarcation potential, threshold, and spike height. The continuous curves are those of the control.

RESULTS

Polarization level and threshold. The stabilizers generally produced a hyperpolarization, usually of less than 2 mV, and a lowered excitability, but differed in their effectiveness and reversibility of action.

Figure 1 demonstrates the typical reversible increase in polarization and threshold induced by low concentrations of

procaine, and the associated negligible change in spike height. Higher concentrations of procaine (e.g., 100 mg %) were observed to cause a gradual decline in spike height which, as noted by Crescitelli ('48) for carbamate, bears little relation to the polarization change; this is to be anticipated from the large "safety factor" in nerve. Cocaine at a concentration of 20 mg % also causes hyperpolarization and a threshold increase; reversibility was not tested.

Yohimbine resembles procaine in causing parallel changes in threshold and polarization, but these are irreversible (Fig. 2A). A detectable increase in threshold occurred with 5 mg %; concentrations of 20 to 40 mg %, in 6 experiments, averaged a 60% increase in threshold, a 24% decrease in spike height, and a 1–2 mV increase in polarization level. The proportionately much smaller alteration of the spike may again be noted.

Pyribenzamine resembled yohimbine in the irreversibility of its effects. Threshold changes occurred at 40 and 100 mg %, and polarization increases were evident at 100 mg %. No definite relation between hyperpolarization and threshold increase could be established. For example, in figure 2B the application of 100 mg % pyribenzamine produces a maximal increment in polarization of about 0.7 mV within 30 minutes, but the threshold continues to increase; when the corresponding control is subsequently subjected to 400 mg %, a negligible increase in polarization occurs, but the threshold undergoes a considerable rise. A slight secondary fall in polarization — perhaps related to that described by Crescitelli for carbamate — is apparent at the higher concentration, but even at 1% it may or may not occur. The spikes disappeared within an hour at 400 mg % and higher, and failed to recover in antihistaminic-free Ringer's.

Preliminary observations were also made with antistine,[3] which has been demonstrated to have anaesthetic activity (Dutta, '49; Yonkmann et al., '49). This antihistaminic[4]

[3] Very kindly provided by Dr. F. F. Yonkmann of Ciba Pharmaceutical Products, Inc.

[4] This term is used merely for convenience; no mechanism of action is implied

caused a small appreciable increase in threshold at 40 mg % accompanied by a depolarization of 1–2 mV.

Veratrine is a depolarizing agent (e.g., Lorente de Nó, '47), causes repetitive activity with stimulation (Welsh and Gordon, '47), and decreases the threshold (Graham, '31). Its action

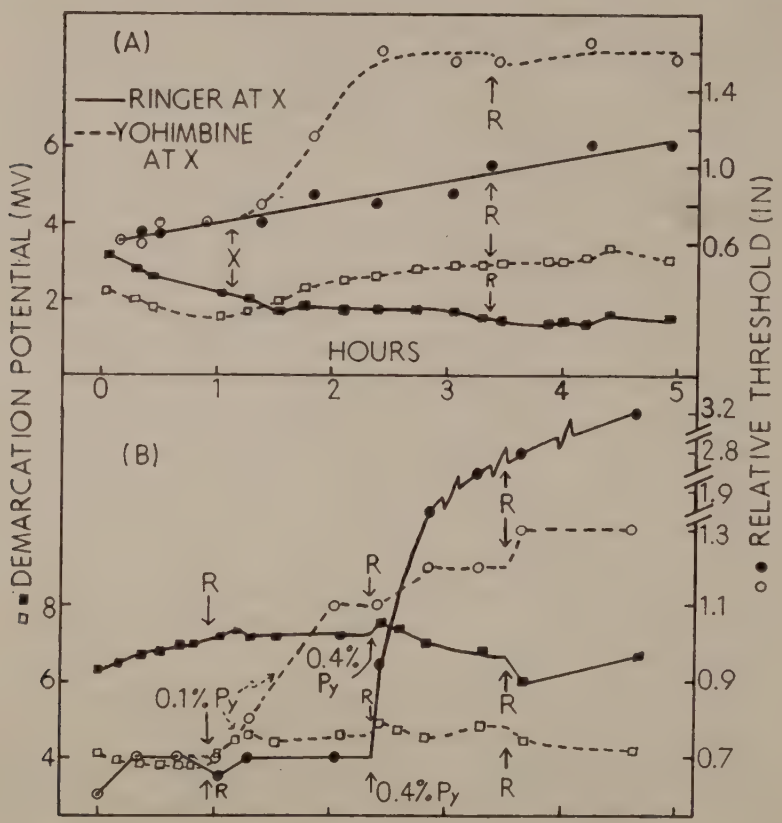

Fig. 2 (A) Effect of 40 mg % yohimbine on threshold (circles) and demarcation potential (squares) compared with control behavior (continuous curves). (B) Corresponding results with 100 and 400 mg % pyribenzamine (Py).

appears to be the opposite of stabilizers; the possibility of an antagonism was therefore explored. Table 1 illustrates that procaine and cocaine, at 30 mg % and higher, exert a striking protective action against veratrine depolarization. The de-

crease in threshold of the order of 30% in an hour, which occurred with veratrine alone, was also prevented by the stabilizers.

The depolarization caused by potassium in nerve and muscle has been shown to be reduced by inorganic multivalent ions (Guttman, '40; Höber and Strohe, '29), and more recently this has been demonstrated for muscle with the organic stabilizers described in the present report (Shanes, '50b). Five experiments with cocaine (10 to 50 mg %) in regular or calcium-free Ringer's failed to produce a consistent change in the amplitude or rate of depolarization by potassium at

TABLE 1

A comparison of the veratrine induced depolarization of control nerves (C) with those of stabilizer treated preparations (X)

STABILIZER	STABILIZER CONCENTRATION	VERATRINE CONCENTRATION	DEPOLARIZATION PER HOUR	
	mg %	*mg %*	C *mV*	X *mV*
Procaine	10	2	3.1	2.5
	30	2	4.6	0
	100	2	2.1	0
Cocaine	10	2.5	3.3	0.9
	30	2.5	3.7	0
	100	2.5	3.0	0

20% of isotonic strength (cf. Shanes, '48a). In 4 experiments with 20 mg % pyribenzamine no consistent effect on potassium action was noted either.

Depolarization by anoxia. Stabilizers reduce the amplitude of the change induced by anoxia, as well as that of the recovery upon return of oxygen.[5] In keeping with the action of unstabilizers such as calcium precipitants (Shanes, '48b), veratrine has the opposite effect (cf. preliminary report, Shanes, '48c).

The data for stabilizers are shown in table 2. Consistent effects were noted with cocaine at concentrations as low as

[5] Such stabilizer action was reported for calcium some time ago (Shanes, '42), and has since been confirmed by Lorente de Nó ('47).

0.5 mg % in strong phoshate buffered Ringer's, and at 1 mg % in ordinary Ringer's. The phosphate medium was frequently employed to accentuate the anoxic depolarization and hence the stabilizer effect; in general the same qualitative effects were to be seen in normal Ringer's, but longer periods of anoxia — at least two hours — were required for significant

TABLE 2

The average depolarization during ca. two hours of anoxia, and the average maximum increment in polarization upon return to oxygen, of stabilizer treated nerves (X) and of controls (C)

STABILIZER	STABILIZER CONCENTRATION	RINGER'S MODIFICATION	DECLINE IN N_2		RECOVERY IN O_2		NO. OF PAIRS
	mg %		C *mV*	X *mV*	C *mV*	X *mV*	
Cocaine	1–6	....	3.0	1.3	3.0	1.8	6
	20–100	....	2.4	1.0	5.5	1.8	6
	0.5–5	25% Ph [1]	6.8	2.1	14.6	6.6	6
	50–100	25% Ph [1]	7.4	2.3	13.6	1.6	3
Procaine	10–100	25% Ph [1]	7.5	3.3	13.7	5.3	3
	1–100	25% Ph [1]	6.5	3.8	16.1	7.1	3
Yohimbine	5	20% Ph [1]	3.9	0.8	9.4	4.0	3
	10	....	2.2	1.0	6.6	3.1	3
	10	20% Ph [1]	9.5	1.7	17.3	6.8	3
	20–40	0.5 × Ca	8.6	4.2	13.7	6.4	4
Pyribenzamine	10–15	....	5.4	1.6	10.6	5.3	4
	40	....	3.7	2.7	6.3	4.6	2
	100	....	2.8	2.6	6.2	4.8	2
	200	....	4.1	0.0	6.6	0.0	2

[1] Calcium absent from both control and experimental solutions, and phosphate buffer of the indicated isotonic strength added.

data. Baseline drift, although usually small, introduced greater variability in the prolonged anoxia experiments; since the recovery in oxygen under most conditons is in proportion to the anoxic decline and occurs rapidly, it provided a further check on the anoxia results.

Table 2 demonstrates that, as pointed out for cocaine, very low concentrations of the stabilizers are effective in decreasng

the change in polarization during anoxia, particularly in the presence of a calcium precipitant. The levels are the same as those which have been shown to affect the threshold. In agreement with this, the very lowest concentrations were seen to stop spontaneous activity induced by high concentrations of phosphate buffer. These concentrations also prevent the repetitive activity set off by an action potential in the presence

TABLE 3

The average depolarization during 1 to 2 hours of anoxia, and the average maximum increment in polarization upon return to oxygen, of veratrine treated nerves (X) and of controls (C). In the experiments with strong phosphate buffer veratrine treatment was preceded by overnight (ca. 16 hours) exposure of both C and X nerves to the indicated modified Ringer's

RINGER'S MODIFICATION	VERATRINE CONCENTRATION	DECLINE IN N_2		RECOVERY IN O_2		NO. OF PAIRS
	mg %	C *mV*	X *mV*	C *mV*	X *mV*	
. . . .	0.2–1.0	3.5	8.4	8.3	13.1	6
0.5 × Ca	0.5	3.6	11.4	9.5	19.7	2
25% Ph[1] + 2 mg % Coc.[2]	0.5–1.0	4.1	6.1	9.3	12.4	6
25% Ph[1] + 2 mg % Coc.[3]	0.5–1.0	6.6	9.9	10.9	14.5	3
50% Ph[1] + 10 mg % Pr[3] + 20 mM Gluc[4]	0.5	4.4	8.2	6.9	11.1	3

[1] See table 2.
[2] No cocaine present during the overnight soaking period.
[3] Stabilizers (cocaine, procaine) present during overnight soaking.
[4] Glucose.

of unstabilizers such as veratrine and phosphate buffer (see below).

Thresholds and spike heights were followed in addition to the demarcation potential during anoxia. As noted by Wright ('46), the threshold increase is delayed, and the spike may begin to decrease before the threshold changes; this is similar to the effect of potassium on these parameters. As previously

pointed out (Shanes, '48a), the inexcitability as well as depolarization during anoxia can be delayed by very low concentrations of stabilizer; higher concentrations, however, which in themselves elevate the threshold, almost invariably cause an early rise in threshold and faster development of inexcitability during oxygen lack.

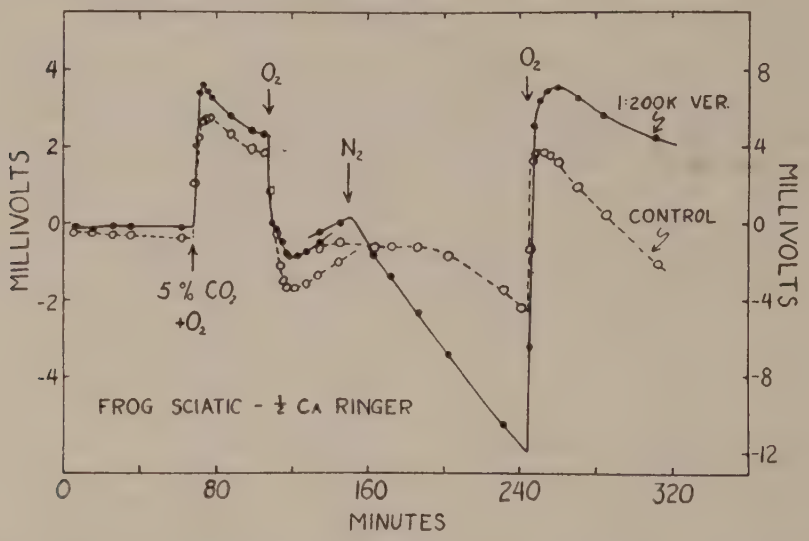

Fig. 3 The potential change of a segment of veratrinized nerve and of a control nerve in 5% CO_2 (scale on the left) and in N_2 (scale on the right) relative to an area kept in O_2. K = 1000.

Typical data demonstrating the acceleration of the anoxic decline of potential in veratrine, and the larger magnitude of recovery in oxygen, are given in table 3 and figure 3. Consistent results are obtained with 0.5 and 1.0 mg %. Even in Ringer's containing 8 times the normal calcium content veratrine caused a more rapid initiation of depolarization in nitrogen. Lorente de Nó ('47) reports an effect by veratrine only on repolarization. However, the use of excessively high concentrations of the alkaloid mixture may have obscured the augmented depolarization during oxygen lack which has been consistently obtained in this research. In the present

study care was taken to employ concentrations which cause negligible polarization changes in oxygen.

In view of evidence for the displacement of calcium from the fiber surface by veratrine (Welsh and Gordon, '47; Shanes, '49a) the effect of pretreatment with a calcium precipitant was explored. Nerves were usually soaked overnight at 4°C. in calcium-free Ringer's containing phosphate buffer at 25 or 50% of isotonic strength; a small quantity of procaine or cocaine was frequently added to prevent spontaneous activity. In all cases good action potentials were obtainable the following day. The representative data in table 3 demonstrate the consistent effectiveness of veratrine under these conditions; moreover, glucose, which itself can reduce the anoxic depolarization. Shanes ('48b) did not prevent the augmentation of the depolarization by veratrine. The rise in threshold and development of inexcitability was also faster in veratrine during anoxia under these various experimental conditions; no initial increase in excitability was noted with oxygen lack when veratrine was present.

DDT is also an unstabilizer (e.g., Welsh and Gordon, '47) and causes repetitive firing in frog nerve (described below). The action of this insecticide on the anoxic deporalization was therefore examined. In 18 paired sets, with suspensions ranging from 0.1 to 10 mg %, chiefly the latter, and with Ringer's containing a normal or half normal calcium content, no consistent effect was noted, although these concentrations were adequate to induce repetitive activity.

Glucose has been demonstrated to delay or prevent anoxic depolarization (Shanes, '48b, '49b); in this respect it resembles the stabilizers. Iodoacetate accelerates the anoxic decline in potential (Shanes and Brown, '42), hence it duplicates the action of veratrine. A further examination of these metabolic agents was therefore of interest. Figure 4 demonstrates the negligible depolarization of glucose treated preparations — in fact a slight enhancement of membrane potential which usually occurs — and the marked inhibition of this glucose effect by iodoacetate. Thus, in 6 experiments, preparations

pretreated with 20 mM glucose averaged a 0.4 mV increase in potential during 2 to 2.5 hours of anoxia, while in the additional presence of 1 mM iodoacetate a decline of 7.6 mV occurred. Usually, the nerves undergoing the greater depolarization show a greater reversal upon return to oxygen; however, as noted previously (Shanes and Brown, '42), the reversal in oxygen of the iodoacetate poisoned tissue, if completed, may require one-half to one hour rather than the few minutes of controls, which is strikingly different from the

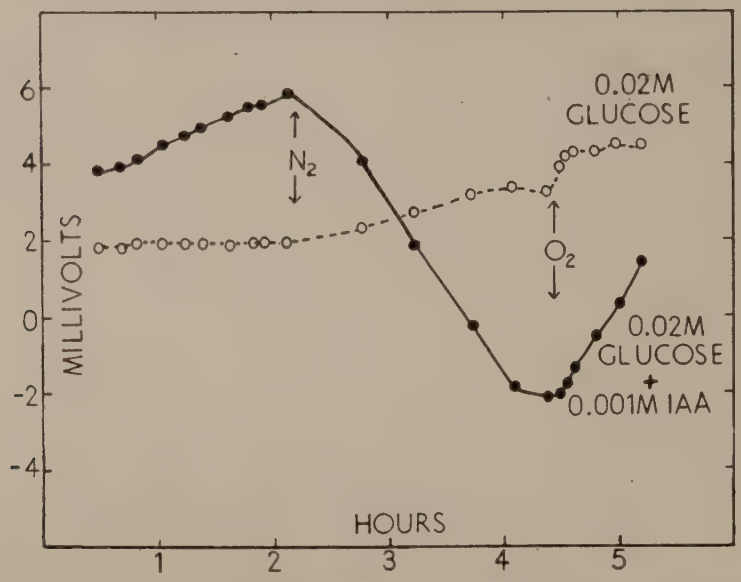

Fig. 4 Demarcation potential changes, in nitrogen and oxygen, of glucose treated nerves with iodoacetate present or absent.

situation with veratrine. Glucose treated nerves showed little failure with respect to the spike in the 2 to 2.5 hours of these experiments; the threshold showed a definite rise, however, which did not subside upon return to oxygen. With the additional presence of iodoacetate, an initial fall in threshold during anoxia was followed by a rapid rise; this and the decline in spike height occurred during the early stages of anoxia. Recovery of spikes and a lowering of threshold fol-

lowed in oxygen. These data confirm and extend the earlier observations; it has not been possible under these conditons to duplicate Lorente de Nó's report ('47) — unfortunately without details — that iodoacetate resembles iodoacetamide in delaying the effects of anoxia.[6]

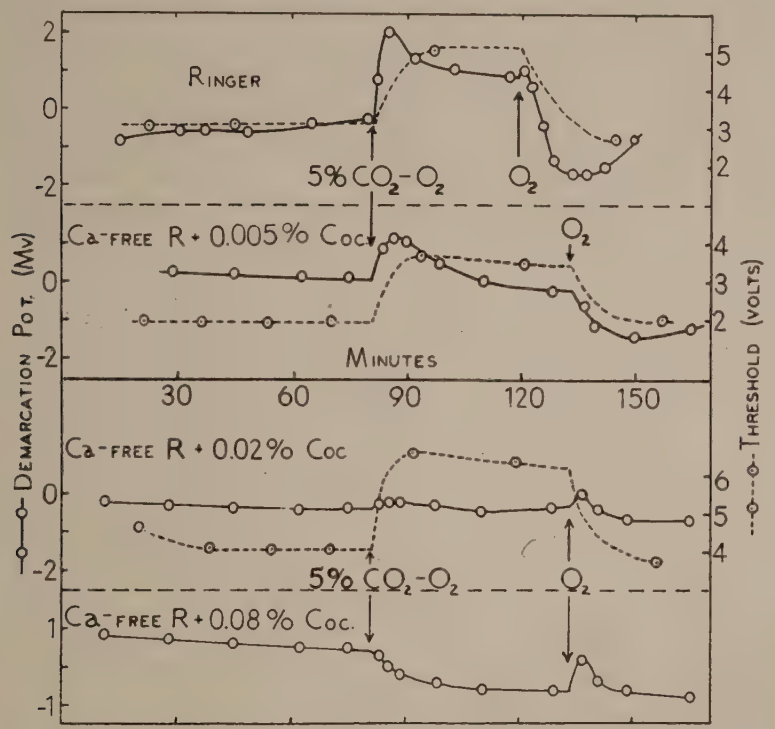

Fig. 5 Polarization and threshold changes upon the application and removal of CO_2 in nerves treated with the indicated solutions for 3.5 hours.

Hyperpolarization by 5% CO_2. All stabilizers examined reduced the amplitude of the response to CO_2. Veratrine caused an overshooting in potential comparable to that obtained in the absence of calcium (Shanes, '48b) but the final level was less than in the controls.

[6] Note added in proof: Feng ('50, Chin. J. Physiol., *17*) has recently shown that the iodoacetamide effect described by Lorente is an artifact and unrelated to metabolic inhibition.

Figure 5 illustrates a typical set of results obtained with graded concentrations of cocaine. It is apparent that although the magnitude of the response to CO_2 is small, its rapidity is such as to permit considerable precision in its measurement, as in post-anoxic recovery. As the stabilizer concentration is increased the amplitude, but not the rate of develop-

TABLE 4

The average maximal increment of polarization upon exposures to 5% $CO_2 - O_2$, and the corresponding reversal upon return to O_2, of controls (C) and of nerves subjected to the indicated concentration of stabilizers (X)

STABILIZER	STABILIZER CONCENTRATION	RINGER'S MODIFICATION	RISE IN CO_2		FALL IN O_2		NO. OF PAIRS
	mg %		C *mV*	X *mV*	C *mV*	X *mV*	
Cocaine	3–6	....	0.9	0.3	1.8	1.2	3
	10		1.4	0.4	1.8	1.1	3
	20		1.9	0.0	2.1	—0.4	2
	50		1.6	—0.7	2.1	—1.0	2
	100		0.8	—0.6	1.1	—0.7	2
	0.5–1	25% Ph [1]	4.2	2.7	4.3	3.6	3
	2.5–5		2.2	0.7	2.8	1.5	3
	50–75		2.6	—1.6	1.8	—0.7	2
	100		2.1	—1.0	1.6	—0.8	2
	200–500		4.2	—1.5	1.9	—0.6	3
Procaine	10	....	3.4	2.2	3.3	2.8	3
	33		3.1	1.1	3.6	1.8	3
	100		3.0	0.8	3.5	1.7	3
Yohimbine	.2	....	3.0	0.5	3.3	1.0	3
	0.5	20% Ph [1]	3.3	1.3	2.8	2.9	3
	1		4.2	2.3	3.8	2.6	3

[1] See table 2.

ment of the augmented polarization in CO_2, is depressed; at higher concentrations the CO_2 response changes to a depolarization. The threshold rises in CO_2 (Lorente de Nó, '47), and has been observed to do so when stabilizers prevented the usual polarization change (fig. 5). The spike usually increased in CO_2 except at the higher stabilizer concentrations. In the latter case a decrease occurred; such a decrease was sometimes observed in controls.

Table 4 lists most of the data gathered for the stabilizers. Except for recovery at the very lowest concentration of yohimbine, the individual pairs contributing to the averages gave the same qualitative results indicated by the averages. It was also noted that the lowest concentration of pyribenzamine (15 mg %) sufficed to cause a marked reversal of the CO_2 effect. These results were obtained whether or not the medium was strongly buffered with phosphate; the latter augments the CO_2 effect (Shanes, '48b), and therefore points up the stabilizer action.

Figure 3 illustrates the typical overshooting of potential which occurred in Ringer's containing veratrine and a normal or half normal calcium content. At calcium concentrations twice above normal and higher, the overshooting in veratrine was prevented; it could then be clearly seen that the final amplitude in veratrine was consistently less than in its absence, an effect usually apparent following the overshooting present at lower calcium concentrations. Thus, in 2x and 8x calcium Ringer's, 6 controls averaged a 70% greater increment than the veratrine treated preparations; this was a highly significant result, for the standard deviation of the differences was one-seventh as great.[7]

The effect on veratrine action of an extreme reduction in the calcium content of the medium — by the use of strong phosphate buffer solutions as described for the anoxia experiments — was also explored. It was found that veratrine produced no consistent effect on the CO_2 response under these

[7] Lorente de Nó ('47) presents data which are interpreted as indicating a greater repolarization by veratrinized nerves in CO_2. Since his experiments are for the most part with depolarizing concentrations of the alkaloid mixture, the difference in results may be attributable to this. However, a technical error may have been misinterpreted. Thus, the more proximal and hence thicker portions of frog nerve normally undergo a greater hyperpolarization in CO_2 than the distal (Shanes, '48b). Lorente applied veratrine to the proximal region and noted that the polarization difference between this and the distal area decreased when the entire nerve was subjected to CO_2; unfortunately, the possibility that the proximal region undergoes a greater polarization even in the absence of veratrine seems not to have been examined. Under the circumstances, therefore, such experiments appear to be of questionable validity.

conditions when 2 mg % procaine was present to prevent repetitive activity. When 10 mg % procaine was present with the alkaloid mixture, a marked depolarization occurred in CO_2, although the controls in a similar medium but lacking veratrine underwent a good hyperpolarization; it is noteworthy that these veratrinized preparations were nevertheless observed to be much more sensitive to anoxia than the controls — the only exception to the otherwise parallel changes in sensitivity to CO_2 and anoxia under the many experimental conditions which have been studied.

DDT, in the same concentrations employed for the anoxia experiments, did not alter the response of the resting potential to CO_2.

Action potential. Under this category are described observations on the negative after-potential following single spikes and on spike and after-potential behavior during repetitive stimulation.

The action of cocaine was studied chiefly. When the spike height of the controls was limited to that of the cocaine-treated nerves, the negative after-potential following single spikes was still appreciably less in the stabilizer. Thus, concentrations ranging from 7.5 to 80 mg % reduced the negative after-potential of 12 nerves by 40%. This is consistent with observations on single fibers of the squid (Shanes, '49a). The notch which frequently follows the spike and which has been considered to be the rising phase of the negative after-potential, is usually larger in cocaine and, because of the smaller negative after-potential, is more likely to overshoot the baseline. This appears to support the conclusion (Lorente de Nó, '47), also reached from a study of the giant axon of the squid (Shanes, '49a), that it is more directly related to a reaction process set off by the spike and comparable to the mechanism concerned with the oscillations initiated by an external electrical pulse. It may therefore be referred to as an "overshoot."

It is well known that a lowered calcium content of the medium leads to spontaneous activity (e.g., Brink et al., '46).

This has been noted during the use of phosphate buffer at concentrations ranging from 20 to 50% of isotonic strength in calcium-free Ringer's. As described previously (see Lorente de Nó, '47, for literature), the initial large response to a single stimulus is followed by a train of impulses decreasing in amplitude. It was noted, too, that the interval between successive spikes increased; in three cases, for example, the interval progressively averaged 3.3, 4.7, and 7 msec. Cocaine, at the lowest concentration examined (7 mg %), prevented both spontaneous firing and the repetitive response. No appreciable negative after-potential was associated with phosphate action. Procaine and yohimbine were also observed to stop spontaneous activity at low concentrations.

Veratrine is particularly noted for its ability to augment the negative after-potential, which may or may not be associated with repetitive activity (Krayer and Acheson, '46). This unstabilizer differs from the calcium precipitants in not giving rise to spontaneous firing. The impulses following a maximal response show a decrement in both the amplitude and the interval between them. Thus, in 4 such preparations three successive intervals averaged 6.3, 4.5, and 4.0 msec, and therefore seemed to be approaching the minimum observed in phosphate. Unlike the situation in the squid axon, the negative after-potential at these veratrine concentrations had a distinct prolonged plateau or slow small rising phase lasting or attaining a maximum at between 25 and 50 msec; this was apparent in veratrinized nerves whether repetitive activity was present or not, or in which the repetitive activity was supressed with low concentrations of cocaine (fig. 6A, B). Repeated stimulation of veratrinized nerves caused a transitory increase followed by a subsidence in the repetitive activity in the wake of individual maximal responses (fig. 6C, D). The increase in the repetitive response is undoubtedly related to the increased oscillatory behavior observed in the squid axon under the same conditions (Shanes, '49a). An additional feature which was particularly clear in tetanus experiments with cocaine and veratrine was the conversion of the slow

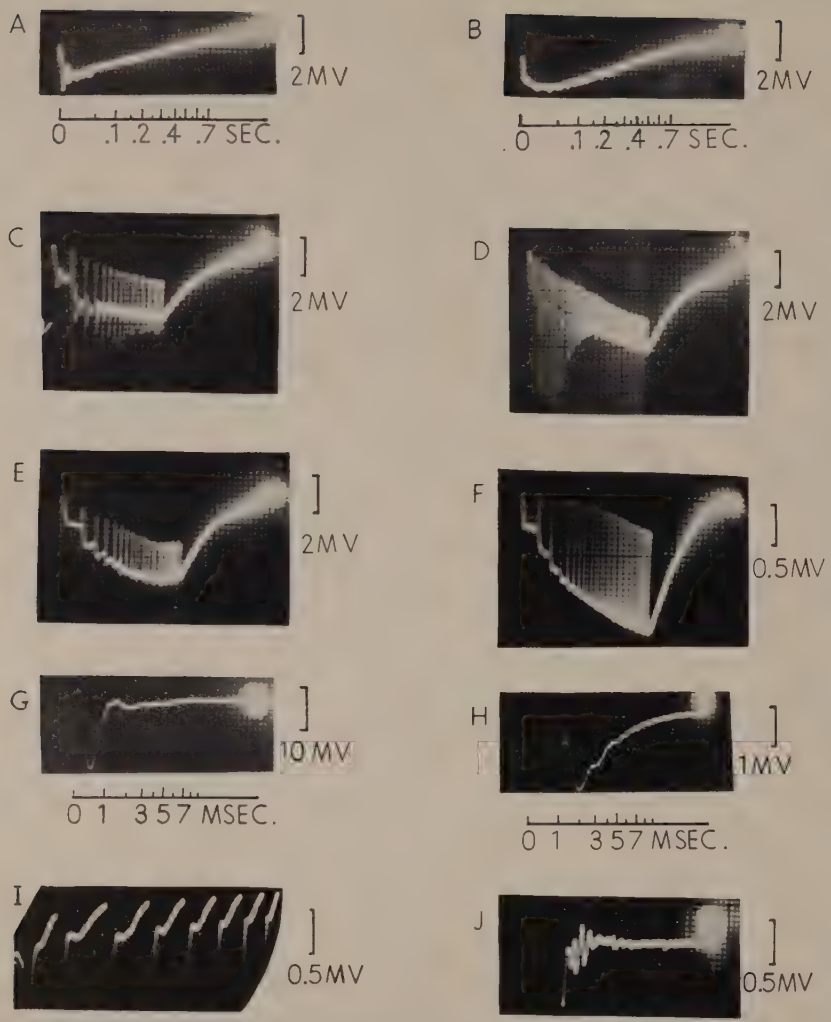

Fig. 6 (A) Negative after-potential and repetitive activity following a maximal response of a nerve subjected to 0.5 mg % veratrine for 7 hours. (B) Negative after-potential of a nerve taken from the same animal as for (A) and similarly treated, but with 3 mg % cocaine present in addition. (C) Negative after-potentials and repetitive activity during a tetanus of 45 shocks per second. (D) Same as for (C), but at a frequency of 150 shocks per second. (E) Summation of negative after-potentials in a nerve previously treated for 6.5 hours with Ringer containing 0.5 mg % veratrine and 3 mg % cocaine; tetanus 45 per second. (F) As in (E) but 16 mM calcium present instead of cocaine. (G) Maximal spike and its following extra response after 3.5 hours exposure to 1 mg % DDT. (H) Same as (G), but higher amplification. (I) Effect of a tetanus of 150 per second on the extra response of a nerve subjected to 10 mg % DDT for 5 hours. (J) Typical extra responses of a nerve exposed to 10 mg % DDT for 19 hours. Each time scale refers only to the figure immediately above it.

rising phase or plateau of the negative after-potential into a decline (fig. 6 E); it could also be seen in the absence of cocaine if the repetitive response was poorly developed. A similar process has already been indicated for Ringer's soaked nerve (see Lorente de Nó, '47, for references). Consistent effects of cocaine on the veratrine induced negative after-potential were not obtained up to 10 mg %; higher concentrations exerted an excessive effect on spike height. Down to 3 mg %, however, the repetitive response was invariably prevented. Calcium at 16 mM concentration, which leaves the spike height about normal, was also observed to prevent the repetitive response; as in cocaine, a plateau or small slow rising phase was characteristic of the initial part of the after-potential. Also, the over-shoot became more apparent during a tetanus, but the slow recovery phase of the negative after-potential did not undergo the marked change seen with cocaine (fig. 6 F).

DDT is considerably less effective than veratrine in inducing repetitive activity in the sciatic, which occupies an intermediate position between the giant axon and crab nerve in its sensitivity to the insecticide. Thus, two to three hours' exposure to DDT at concentrations from 0.1 to 10 mg % characteristically caused the appearance of a single, more or less distinct extra response following a stimulus (fig. 6 G, H); this was $7 \pm 1\%$ of the amplitude of the maximal response and occurred 2.4 ± 0.1 msec later. It disappeared immediately with repetitive stimulation (fig. 6 I). The peak of the second spike coincided closely with the time of the maximum of the negative after-potential. Penetrability probably was a limiting factor in DDT action, for preliminary soaking at low temperature for 17 hours increased the number of repetitive impulses, but theses were of small amplitude (fig. 6 J); spontaneous activity was frequently observed under these conditions. Under no circumstances, however, was an appreciable negative after-potential obtained — a situation quite reminiscent of that in crab nerve (Shanes, 49b).

DISCUSSION

The correlations and interrelations established through modifications of threshold, of repetitive activity, and of anoxia and CO_2 effects on the resting potential by the same concentration levels of stabilizers and unstabilizers, are presumptive evidence for a common controlling factor. In addition, such observations provide a variety of conditions for testing any hypothesis which may be put forward.

Metabolic alterations, suggested again recently by Lorente de Nó ('47), are an unlikely basis for the interpretation of these results because the concentrations involved, at least in the case of some of the stabilizers (Sherif, '30), are far below those known to have a respiratory effect. Potassium shifts, on the other hand, which were predicted from these and related studies (Shanes, '48b, '49b; Shanes and Hopkins, '48) have been (Fenn and Gerschman, '50; Shanes, '50 a, c) and continue to be confirmed (unpublished)[8] as the major factor directly responsible for at least the slower bioelectrical fluctuations in nerve. It is beyond the scope of the present report to discuss this in detail; in summary, however, it may be stated that to date it has been found that within the range of conditions studied, wherever a depolarization is known to occur or to become augmented a potassium release occurs or is enhanced and, conversely, when depolarization is retarded or the polarization level elevated, the corresponding opposite effects on potassium movement are to be seen. The establishment of this fact at least with respect to the resting potential is of importance in elucidating the mechanism of bioelectrical changes and of the action of agents such as those studied in this report; it is also important in that electrical data may supplement chemical measurements in the clarification of problems related to potassium accumulation and

[8] In a report on "Potassium movement in relation to drug and ion action in nerve," presented June 21, 1950 at a symposium on electrolytes conducted at the Marine Biological Laboratory in Woods Hole by the Society of General Physiologists, further correlations were described for veratrine, glucose, iodoacetate, sodium and activity ('50, Biol. Bull., *99:* 309).

sodium exclusion; and, finally, the additional relationships which have been described have important implications for the action potential and excitability phenomena.

The many conditions leading to an elevated threshold make clear that it is not necessarily associated with an increased polarization level. The parallelism which may be obtained with procaine is suggestive of a common site of action, but a secondary effect (possibly metabolic inhibition associated with interference with the potassium retention machanism) apparently may prevent it in the case of other stabilizers. None of the agents tested increases the polarization level of tissues which are much more permeable to chloride than frog nerve (Guttman, '40; Shanes, '50a, b). Muscle differs from vertebrate nerve further in that the depolarizing action of KCl is markedly reduced by these stabilizers (Shanes, '50b).

The failure of the nerves to recover from yohimbine and pyribenzamine cannot necessarily be considered evidence for "toxicity" in the sense of destructive action; for example, these agents may combine much more firmly with fiber structure than does procaine. The maintenance of the resting potential, the depolarization still induced by CO_2, and the continued effectiveness of KCl as a depolarizing agent, support this view. Such irreversible or poorly reversible combination, if it occurs, ultimately may prove useful for the elucidation of the site of action.

A change of threshold does not necessarily reflect a true change of the individual fibers. Thus, it may merely indicate a failure of the more excitable units; the observation that the spike height frequently was not depressed in CO_2 is suggestive of a real threshold change in this gas. The localized application of stabilizers introduced an additional possible extraneous factor, viz., a shift in the point of excitation away from the cathode. Better controlled conditions are obviously necessary for an adequate evaluation of the threshold data.

The results obtained with veratrine, both in nitrogen and CO_2, are further in accord with the view that this alkaloid displaces calcium from the fiber surface. The increased

oscillatory activity indicated by the initial augmented repetitive firing during a tetanus, comparable to the situation in squid axon, suggests that veratrine competes more effectively with the surface calcium during activity. However, a more complex situation under certain conditions is indicated by its stabilizer effect with respect to CO_2 action and its unstabilizer effect in the absence of oxygen. As an alkaloid mixture veratrine would be expected to resemble other stabilizing alkaloids (e.g., cocaine, yohimbine), which may act by virtue of their considerable positive charge (Shanes, '50b); the smaller final amplitude of the hyperpolarization in CO_2 in high calcium Ringer's may also be an indication of such stabilization. If such a stabilizing effect is present, it is usually masked by the more specific unstabilizer action.

DDT may compete for calcium far less effectively than veratrine, to which its low water solubility would contribute. The absence of an augmentation in repetitive response during a tetanus is an important feature which may be related to the lack of an increase in negative after-potential in the insecticide. In any event the differences between DDT and veratrine indicate, as they did in crab nerve (Shanes, '49b), that the negative after-potential is correlated with the augmented depolarization during oxygen lack and, moreover, that the negative after-potential is not a necessary condition for repetitive firing.

The similarity between glucose and stabilizers with respect to anoxic depolarization is of interest in view of a recent report of glucose potentiation of barbiturate anaesthesia (Lamson et al., '49); the rise it causes in threshold during anoxia probably is also pertinent. Although this substrate and the stabilizers act similarly in certain respects, including an improvement in potassium retention during anoxia (Shanes, '50a, c), the mechanism may differ; the former probably acts metabolically, the latter physically. Two indications of the basic difference in the glucose mechanism are (a) the absence of a marked threshold effect in oxygen and (b) the maintained activity during several hours of anoxia.

The author is indebted to Miss Tess Abramsky for many of the measurements which have been described.

SUMMARY

Stabilizers increase the resting potential, delay depolarization during anoxia, and decrease the hyperpolarization in 5% CO_2. Veratrine, classified as an "unstabilizer," resembles calcium precipitants in exerting opposite effects; DDT, in concentrations sufficient to cause weak repetitive activity, had no such action. Effective concentrations are found to be the same as those which modify threshold and the action potential, from which it is suggested that one labile factor, common to all these phenomena, is involved.

LITERATURE CITED

BENNETT, A. L., AND K. G. CHINBURG 1946 The effects of several local anaesthetics on the resting potential of isolated frog nerve. J. Pharmacol. and Exp. Therap., *88:* 72.

BISHOP, G. H. 1932 Action of nerve depressants on potential. J. Cell and Comp. Physiol., *1:* 177.

BRINK, F., D. W. BRONK AND M. G. LARRABEE 1946 Chemical excitation of nerve. Ann. New York Acad. Sci., *47:* 457.

CRESCITELLI, F. 1948 The dual action of carbamates on the resting potential of frog nerve. J. Cell. and Comp. Physiol., *32:* 187.

DUTTA, N. K. 1949 Some pharmacological properties common to antihistaminic compounds. Brit. J. Pharmacol. and Chemotherap., *4:* 281.

FENN, W. O., AND R. GERSCHMAN 1950 The loss of potassium from frog nerves in anoxia and other conditions. J. Gen. Physiol., *33:* 195.

GRAHAM, H. T. 1931 Antagonism of alkaline earth to alkali metal cations as manifested in changes of the after-potential of nerve. Pharmacol. and Exp. Therap., *42:* 269.

GUTTMAN, R. 1940 Stabilization of spider crab nerve membranes by alkaline earths, as manifested in resting potential measurements. J. Gen Physiol., *23:* 343.

HÖBER, R., AND H. STROHE 1929 Über den Einfluss von Salzen auf die elektrotonischen Ströme, die Erregbarkeit und das Ruhepotential des Nerven. Arch. ges. Physiol., *222:* 71.

KRAYER, O., AND G. ACHESON 1946 The pharmacology of the veratrum alkaloids. Physiol. Rev., *26:* 383.

LAMSON, P. D., M. GRIEG AND B. H. ROBBINS 1949 The potentiating effect of glucose and its metabolic products on barbiturate anaesthesia. Science, *110:* 690.

LORENTE DE NÓ, R. 1947 A study of Nerve Physiology. New York, The Rockefeller Institute for Medical Research, Parts I and II.

SHANES, A. M. 1942 A metabolic basis for the stabilization of resting potentials by calcium. J. Cell. and Comp. Physiol., *19:* 249.

———— 1948a An experimental and theoretical approach to the mechanism of cocaine action. Science, *107:* 679.

———— 1948b Metabolic changes of the resting potential in relation to the action of carbon dioxide. Am. J. Physiol., *153:* 93.

———— 1948c The effect of "stabilizing" and "unstabilizing" agents in relation to the metabolic mechanism supporting the resting potential of nerve. Biol. Bull., *95:* 245.

———— 1949a Electrical phenomena in nerve. I. Squid giant axon. J. Gen. Physiol., *33:* 57.

———— 1949b Electrical phenomena in nerve. II. Crab nerve. J. Gen. Physiol., *33:* 75.

———— 1950a Potassium retention in crab nerve. J. Gen. Physiol., *33:* 643.

———— 1950b Drug and ion effects in frog muscle. J. Gen. Physiol., *33:* 729.

———— 1950c Potassium shifts in nerve. Fed. Proc., *9:* 116.

SHANES, A. M., AND D. E. S. BROWN 1942 The effect of metabolic inhibitors on the resting potential of frog nerve. J. Cell. and Comp. Physiol., *19:* 1.

SHANES, A. M., AND H. S. HOPKINS 1948 Effect of potassium on "resting" potential and respiration of crab nerve. J. Neurophysiol., *11:* 331.

SHERIF, M. A. F. 1930 The effect of certain drugs on the oxidation processes of mammalian nerve tissue. J. Pharmacol. and Exp. Therap., *38:* 11.

WELSH, J. H., AND H. T. GORDON 1947 The mode of action of certain insecticides on the arthropod nerve axon. J. Cell. and Comp. Physiol., *30:* 147.

WRIGHT, E. B. 1946 A comparative study of the effects of oxygen lack on peripheral nerve. Am. J. Physiol., *147:* 78.

YONKMANN, F. F., F. ROTH, J. SMITH, N. HANSEN AND B. N. CRAVER 1949 Local anaesthetic properties of antistine and pyribenzamine hydrochloride. Anaesthesia and Analgesia, *28:* 170.

THE EFFECT OF THYROID HORMONE AND RADIATION ON THE MITOTIC INDEX OF MOUSE EPIDERMIS

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FOUR FIGURES

The transitory arrest of mitosis in the epidermis of the mouse ear has been shown by Knowlton and Hempelmann ('49) to be a sensitive index of radiation dosage to the whole body in the range of 5 to 325 r. Administration of the thyroid hormone has been reported to cause an increase in the mitotic activity of cells of the adrenal gland (Schmidt and Schmidt, '38) and to cause an enlargement of kidney, spleen and heart (MacKay and MacKay, '31). Since thyroid administration to mice after whole-body irradiation considerably increases mortality (Smith and Smith, '51a) it occurred to us that the effect of thyroid on recovery of mitotic activity after the arrest caused by radiation should be investigated.

PROCEDURES AND RESULTS

The general procedures concerning maintenance and irradiation of animals have been described elsewhere (Smith and Smith, '51a). Only male mice of N.I.H. stock were used; they were irradiated at the age of 7 to 8 weeks; litters and irradiation groups were distributed as evenly as possible between treatment groups; and the mice were caged individually after irradiation. Thyroid treatment consisted of the addition of U.S.P. desiccated thyroid to the diet of finely ground pellets in a concentration of 0.3%. The radiation dose

in the first two experiments was 325 r (air), other radiation factors being: 170 Kv, 20 ma., 0.25 mm copper and 0.51 mm aluminum added filtration, 50 cm focal distance, and dose rate 56 to 62 r per min. In the third experiment the dose was 400 r and the only added filtration 0.08 mm of copper.

The mice on the whole tolerated the diet containing thyroid very well. Of several hundred mice so treated only 3.6% died in 28 days and the average gain in body weight was almost 10% above normal. The radiation dose of 325 r was sublethal to about 95% of the mice that were not given thyroid, and to about 80% of those given the thyroid treatment (Smith and Smith, '51a).

Sampling of the two ears of a mouse was always separated by at least one week. The samples were always taken between 9 and 11 o'clock in the morning to minimize variation due to diurnal cycles. The ear was quickly wiped with alcohol, about two-thirds of it snipped off, the margins trimmed, and the samples dropped into 1% acetic acid and placed in the refrigerator at about 5°C. Preparation and study of the tissue followed in general the technique outlined by Knowlton and Hempelmann. In our hands aceto-carmine stain gave more satisfactory results than hematoxylin. The stain was prepared by directions given in Lee ('37), the desired amount of iron being reached by trial. The tissue, epidermis down, was floated on the stain for 5 to 20 minutes, and with a glass section lifter transferred through a series of alcohols to be cleared in a mixture of equal parts of acetic acid, alcohol and xylol, then xylol-methyl benzoate and methyl benzoate. A green filter was used in the microscope lamp.

We also endeavored to follow the procedure of Knowlton and Hempelmann in determining the mitotic index. Counts were made by 7 individuals in the course of the studies, and for the sake of uniformity cells were counted only from late prophase to completion of the new cell membrane. The average number of cells encompassed in the Miller disc was so near 200 that this was taken to be the number scanned in each square. The determination of index consisted of the scanning

of an estimated 40,000 cells, and each point in an experiment represents the average of 5 indices. The points of figures 1 and 2 represent averages of two experiments, or the scanning of 400,000 cells from 10 mice. The average of 123 determinations of normal mitotic index in these experiments was 151 $\pm$ 8 cells per 100,000, which agrees closely with the value.

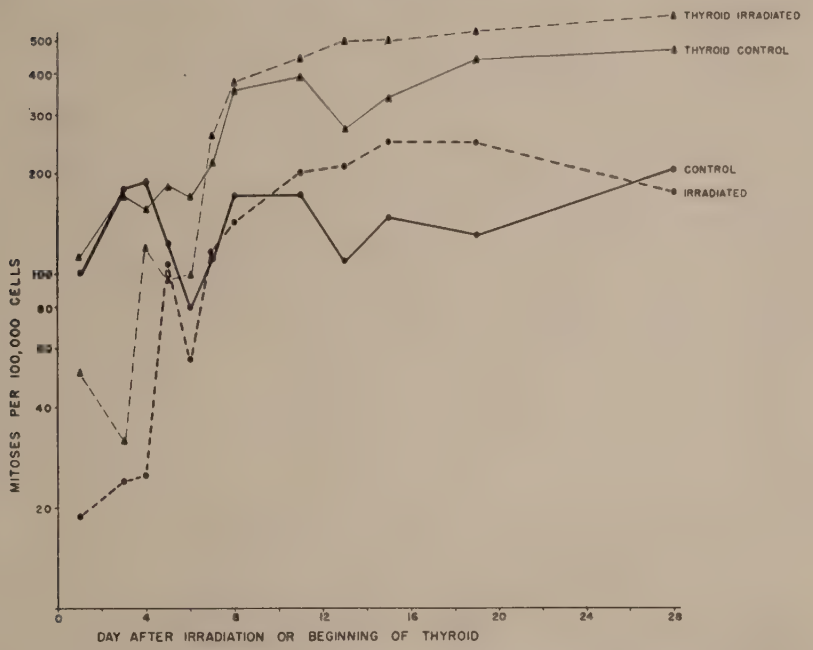

Fig. 1 Mitotic index of ear epidermis after irradiation with 325 r, with and without addition of 0.3% thyroid to the diet, compared with corresponding control values.

169 per 100,000 reported by Knowlton, Hempelmann and Hoffman ('48).

The mitotic index of the thyroid-treated mice was approximately twice normal after about a week on the 0.3% thyroid diet (fig. 3). The development of this thyroid effect reached a plateau at approximately the same time as other thyroid effects observed under similar experimental conditions (Smith and Smith, '51b).

The mitotic index of the ear epidermis after 325 r to the entire body was depressed for about 4 days, returning to near normal on the 5th day and subsequently exceeding normal. This sequence agrees very well with that described by Knowlton and Hempelmann. In the mice fed thyroid the percentage change in mitotic index after radiation was remarkably similar (fig. 2).

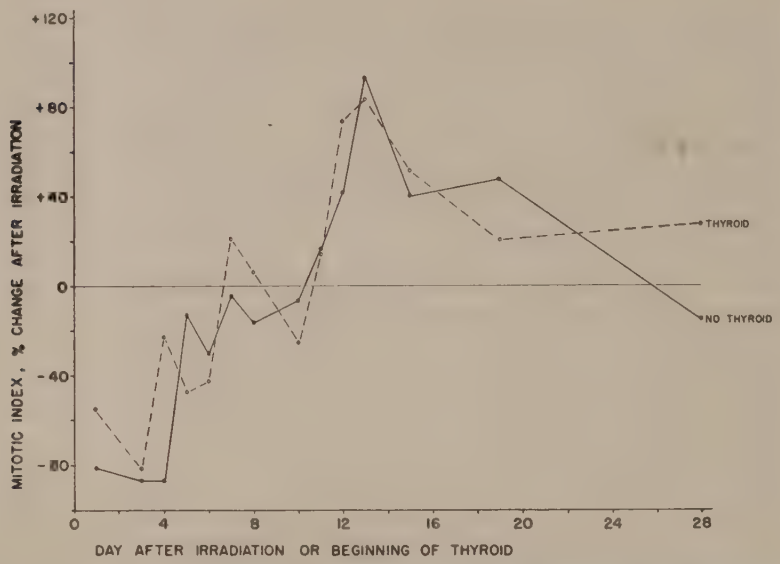

Fig. 2 The per cent change in mitotic index of ear epidermis after irradiation with 325 r, with and without addition of 0.3% thyroid to the diet, relative to corresponding control values.

Analysis of variance [1] of the two experiments combined, involving counts on 440 ears in 4 treatment groups, shows that thyroid had no significant effect on the response to radiation, and conversely that radiation had no effect on the response to thyroid. Both the response to radiation and that to thyroid were significant. The standard deviation of one point which represents the mean of 10 indices, is 38% of this mean. Sources of variation between experiments are respon-

[1] We are indebted to Mr. Nathan Mantel of the National Cancer Institute for this analysis.

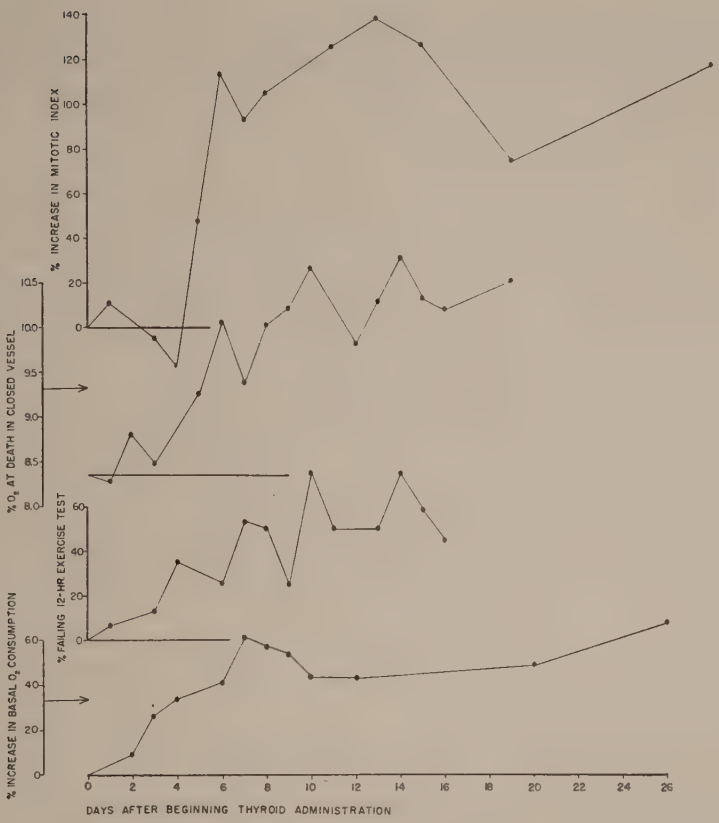

Fig. 3 The effect of the addition of 0.3% thyroid to the diet on mitotic index, sensitivity to progressive hypoxia, tolerance to exercise, and basal O_2 consumption.

sible for about one-fourth of the variance, and variation between individual ears plus counting error for the remainder.

In the third experiment thyroid feeding was begun 16 days prior to irradiation so that the mitotic index would be elevated at the time of irradiation. The mean mitotic index of 33 control ears was 107 ± 10, and of 53 ears of thyroid-fed mice 246 ± 23. Samples were taken as shown in figure 4. The groups were imperfectly balanced [2] and results from

[2] In a number of preparations the epidermal cells floated away from the dermis to such an extent that counts could not be made. The cause is not clear, but may possibly have been a failure to cool promptly enough the acetic acid in which the ears were placed.

irradiated control and thyroid-fed mice are expressed as per cent deviation from the general means of non-irradiated control and thyroid-fed mice respectively, rather than from the corresponding daily means.

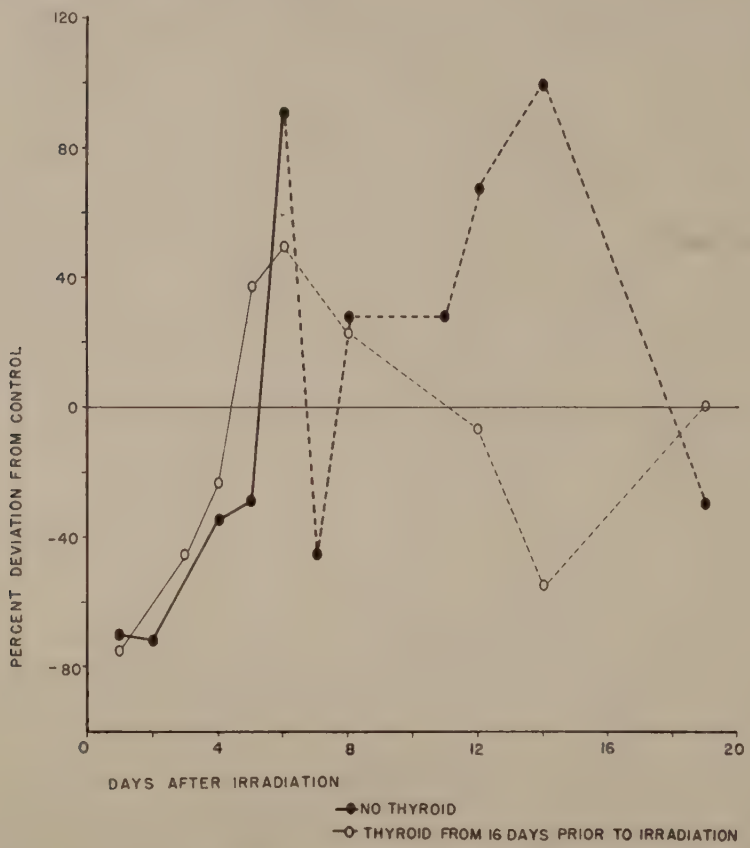

Fig. 4 The per cent change in mitotic index of ear epidermis after irradiation with 400 r, with 0.3% thyroid in the diet beginning 16 days prior to irradiation and without added thyroid, relative to corresponding control values.

For 6 days after irradiation the percentage change in mitotic index of the thyroid-fed mice followed closely that of the mice not given thyroid. It is impossible to evaluate subsequent irregularities in the two groups because the number of samples studied was small relative to the magnitude of the

variability encountered. It may be that a wide range of irregularity generally follows interruption of the normal mitotic rhythm.

DISCUSSION AND CONCLUSIONS

In our experience variability between individual mice and between experiments is such that large numbers of determinations are required for accurate evaluation of effects. Bullough ('48a, '48b, '49) has made extensive studies of environmental factors which affect mitotic acivity. We made no attempt to follow diurnal variations, but chose a time for sampling which roughly coincides with the maximum reported by Cooper and Franklin ('40).

We have not been able to find in the literature any reference to a general increase in mitotic index accompanying thyroid administration, although enlargement of certain organs has been known for many years. In our mice given thyroid in a concentration of 0.3% in the diet the mitotic index of ear epidermis sampled between 9 and 11 A.M. was found to increase to a value approximately twice that of the control group and to show little if any change after the first week. The time at which the full effect is developed corresponds closely with that of other thyroid effects, including the increase in Q_{O_2} and in sensitivity to progressive hypoxia and the decrease in tolerance to exercise. It seems to us extremely interesting and significant that the development of these diverse manifestations of thyroid action should follow such closely similar time curves.

The increased metabolic activity accompanying thyroid administration is associated with an increased susceptibility to the lethal effects of radiation. The mitotic index of the ear epidermis of the thyroid-fed mouse is not, however, abnormally subject to the effect of radiation. This appears to be true both when irradiation is preceded by 16 days of thyroid feeding and when feeding is begun immediately after irradiation. It is equally true of the percentage deviation from control and the duration of depression of the index, within the

limits of accuracy of our experiments. We do not know any basis upon which this result could have been predicted with confidence.

Had the depression of the index in thyroid-treated animals been more extensive in degree or duration one might have suspected a causal relationship to exist between such a response and the effect of thyroid or radiation lethality. At present no particular manifestation of thyroid action has been shown to be more closely associated than any other with the thyroid effect on radiation lethality.

Although thyroid increases radiation lethality, it was found that radiation does not affect numerous factors known to be involved in the action of thyroid. Thus thyroid causes a modification in the response of the mouse to radiation, while radiation does not appear at this time to modify grossly the response of the mouse to thyroid.

SUMMARY

1. Thyroid administration caused an increase in the mitotic index of mouse ear epidermis sampled in mid-morning, which reached full development at the same time as other manifestations of thyroid action.

2. Thyroid administration did not affect the response of mitotic activity, as measured by the mitotic index of the mouse ear epidermis, to sub-lethal whole-body irradiation. Conversely, radiation did not affect the response of mitotic activity to thyroid administration.

LITERATURE CITED

BULLOUGH, W. S. 1948a Mitotic activity in the adult male mouse, *Mus musculus* L. The diurnal cycles and their relation to waking and sleeping. Proc. Roy. Soc., B, *135:* 212–233.

———— 1948b The effect of experimentally induced rest and exercise on the epidermal mitotic activity of the adult male mouse, *Mus musculus* L. Proc. Roy. Soc., B, *135:* 233–242.

———— 1949 The effects of high and low temperatures on the epidermal mitotic activity of the adult male mouse, *Mus musculus* L. J. Exp. Biol., *26:* 76–82.

COOPER, Z. K., AND H. C. FRANKLIN 1940 Mitotic rhythm in the epidermis of the mouse. Anat. Rec., *78:* 1–8.

KNOWLTON, N. P., JR., AND L. H. HEMPELMANN 1949 The effect of x-rays on the mitotic activity of the adrenal gland, jejunum, lymph node and epidermis of the mouse. J. Cell. and Comp. Physiol., *5:* 73–92.

KNOWLTON, N. P., JR., L. H. HEMPELMANN AND J. G. HOFFMAN 1948 The effects of x-rays on the mitotic activity of mouse epidermis. Science, *107:* 625–626.

LEE, BOLLES 1937 The Microtomist's Vade-Mecum. 10th ed. J. A. Curchill Ltd., London, 1937.

MACKAY, E. M., AND L. L. MACKAY 1931 Factors which determine renal weight. X. The effect of feeding desiccated thyroid. J. Nutr., *4:* 33–37.

SCHMIDT, I. G., AND L. H. SCHMIDT 1938 Variations in the structure of adrenals and thyroids produced by thyroxine and high environmental temperatures. Endocr., *23:* 559–565.

SMITH, W. W., AND F. SMITH 1951a The effect of thyroid hormone on radiation lethality. Am. J. Physiol., *165:* 639–650.

———— 1951b Effects of thyroid and radiation on sensitivity to hypoxia, basal rate of O_2 consumption, and tolerance to exercise. Am. J. Physiol., *165:* 651–661.

THE RELATION OF MITOSIS TO THE MANIFESTATION OF X-RAY DAMAGE IN HEMATOPOIETIC CELLS OF TADPOLES [1]

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FIVE FIGURES

It has previously been demonstrated by this laboratory (Allen et al., '50) that the visible destruction by 500 r hard x-rays in the hematopoietic cells of tadpoles of Rana catesbiana is dependent upon and proportional to the post x-ray environmental temperature of these animals.

Since tadpoles are poikilothermic and are thus dependent upon environmental temperature for metabolic function, these findings suggest metabolic activity as an important factor in the manifestation of destruction by this dosage of x-rays. Moreover, since evidence exists that rate of mitosis is also proportional to temperature, metabolic activities associated with cell division may be those specifically involved.

Thus we must consider the possibility that these cells show visible damage from previously administered x-rays only upon entering into mitosis.

The present work is a quantitative test of the above concept. In it we have utilized the mitotic inhibitor colchicine to give an index of rate of division in tadpole hematopoietic tissue at different temperatures. We find that the post irradiation mitotic activity in these tissues is, indeed, quantitatively proportional to cell destruction by 500 r at each of the different temperatures employed.

[1] This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

MATERIALS AND METHODS

1. Tadpoles of Rana catesbiana were matched as to size and divided into three like groups (70 to 80 mm length; 10 to 5 gm weight).

2. These groups were treated as follows:

Group A. 500 r x-irradiation only. (X-irradiation treatment was given with a Picker industrial unit. The irradiation factors were 35 cm T.O.D., 15 ma, 0.21 mm Cu + 0.5 mm parabolic Cu + 1.0 mm Al filters, H.V.L. = 2.1 mm Cu roentgens measured in air.)

Group B. 10 γ to 20 γ colchicine injected into the tail.

Group C. 500 r x-irradiation plus 10 γ to 20 γ colchicine injected into the tail.

3. The tadpoles were kept for 24 hours post-treatment at certain selected temperatures (varying from 4.5°C to 21°C) and were then sacrificed.

4. After killing, the kidneys, which contain a large portion of the hematopoietic cells in these amphibians, were immediately excised and fixed in Bouin's fluid. These were later stained with either Leishman's or Giemsa's blood stains.

5. On the stained material, the percentage of destruction and/or division sustained by the tissue was determined by tallying the damaged or mitotic versus the non-damaged or non-mitotic cells within a microscopic field defined by a Whipple grid.

6. Comparisons were then made between the three groups of experimental animals and were recorded in the results below.

RESULTS

Figure 1 illustrates the strikingly close correlation between amounts of mitosis in 24 hours and x-irradiation destruction during this same period by 500 r at various temperatures. Both are, of course proportional to temperature. When colchicine and 500 r x-irradiation are applied simultaneously, the total percentage cell alteration during the 24 hour period does

not exceed destruction by irradiation alone or stoppage of mitosis by colchicine alone.[2]

It should be mentioned that the application of colchicine did not always result in a pure definition of mitotic inhibition.

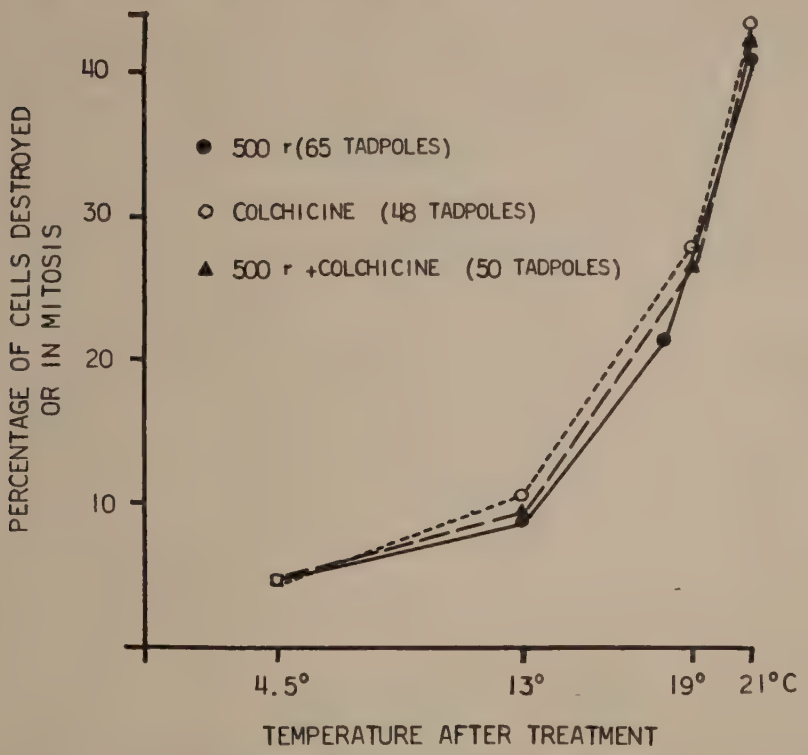

Fig. 1 Relationship between rate of mitosis and x-ray destruction at various temperatures. At point A (4.5°C) the alterations in the three groups are within 0.4%. All tadpoles were killed 24 hours post-treatment.

Indeed, many cells appeared to be destroyed by colchicine rather than merely inhibited. However, in the preceding graph the colchicine curve represents, not only percentage

[2] Work subsequent to that above has demonstrated that in the case of 500 r *plus* colchicine there is an initial acceleration in rate of cell destruction over the alterations produced by either agent when used alone. However, by the time 24 hours have passed these rates have adjusted themselves in accordance with our findings above. A publication is under preparation which will deal with this in detail.

of mitosis, but that of the sum of the destroyed cells and those more obviously in the mitotic state, on the assumption that the destroyed cells were also dividing when affected by colchicine. The validity of this assumption will be discussed later.

Further affirmation of the relationship between post irradiation mitosis and x-ray destruction as demonstrated in figure 1, is given in figures 2 and 3. In these experiments the groups of animals were treated similarly to those in figure 1,

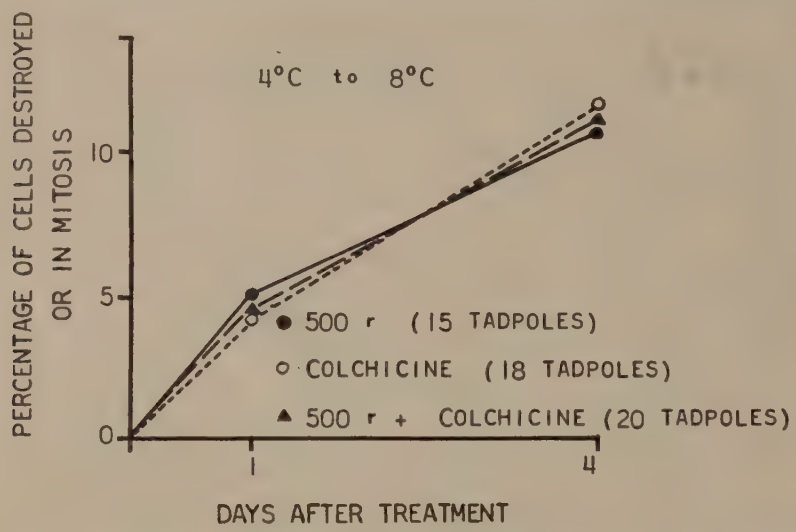

Fig. 2 Relationship between percentage of x-ray destruction and percentage of mitosis over a 4 day post-irradiation interval. The temperature varied from 4°C to 8°C.

but the sacrifices were made over a greater interval of time, thus permitting a more critical examination of the relationship.

HISTOLOGICAL OBSERVATIONS

The nature of the histological alterations in the three experimental groups is described below:

Group A — 500 r x-irradiation only. Typical destruction in the hematopoietic tissues of this group includes nuclear pyknosis, homogenization, and fragmentation. Given time this

injury will progress to extensive nuclear scatter and cytoplasmic disintegration followed by cytolysis. Alterations in "Mulberry" type nuclei (nuclei composed of aggregated chromosomal vesicles) are also observed. These are intact but have an accentuation of stain bordering the periphery of each vesicle as opposed to these types of cells in control animals.

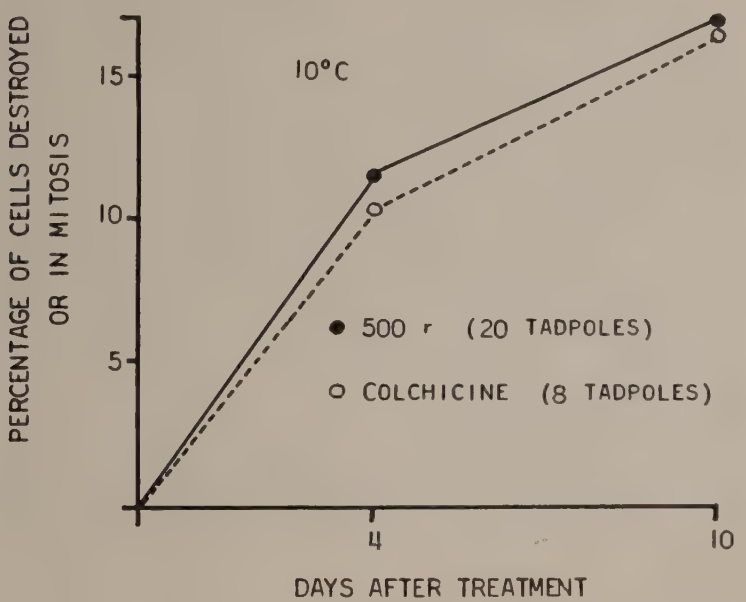

Fig 3 Relationship at 10°C between percentage of x-ray desruction and percentage of mitosis over a 10 day post-treatment interval.

In many cases degenerating cells occur in groups of two or more. Rarely are mitotic figures seen at the higher temperatures but some cells in prophase are observed in experiments performed at 4.5°C.

Group B — colchicine only. Those cells which are arrested in obvious mitosis are observed to be in the early metaphase as contrasted to the usual "condensed metaphase" condition which has long been associated with the administration of colchicine. Bergner ('50) also noted this type of inhibition

following strong colchicine doses. Some typical inhibition patterns are depicted in figure 4.

It has already been stated that colchicine, as administered in the dosages quoted in this work, results in destruction of cells as well as inhibition of mitosis. Bergner ('50) has shown that spermatagonia inactivated by strong doses of

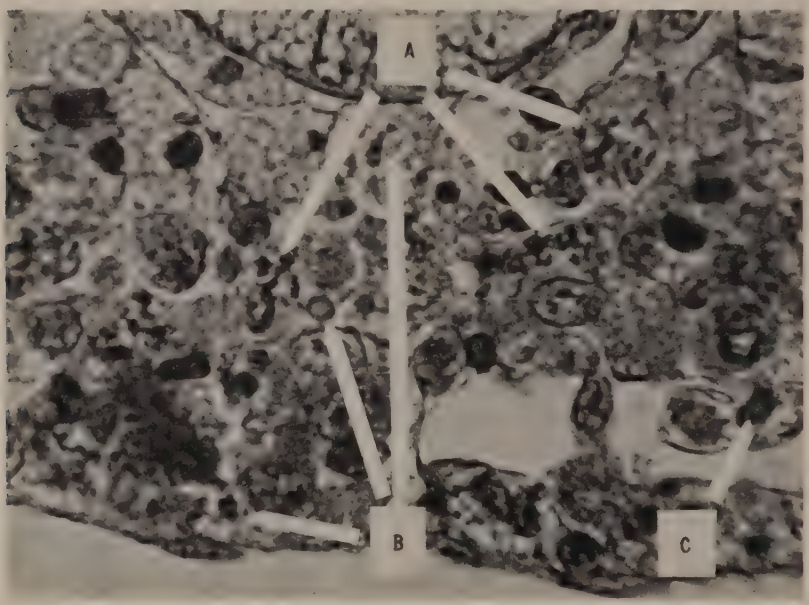

Fig. 4 Some typical effects of colchicine as observed in the hematopoietic tissue of tadpoles 24 hours post-injection. The arrows originating at "A" point to cells which have been arrested in the early metaphase. This stoppage seems to be characteristic of the dosages employed. Arrows from "B" indicate cells which have been destroyed by colchicine and "C" shows a normal condensed nucleus of a red blood cell.

colchicine proceed in time to very similar destruction, a condition characterized by pycnotic nuclei or even pale amorphous masses (fig. 4). Thus it appears that destroyed cells also provide a criterion for mitotic activity.

That destruction by colchicine is actually related to mitosis is further shown by the results of an experiment in which all factors were kept constant except the dosage of colchicine

administered. Animals which received a lower dosage of 10 γ colchicine showed 30% more mitotic figures than those receiving 20 γ colchicine. However, in these two cases the sum of the destroyed cells, plus those stopped in mitotic phases, was 45.7% and 42.0% respectively. It is evident that the dosage of colchicine employed will cause a variation in the number of persistent mitotic figures, but will not significantly alter the index of rate of mitosis if the sum of destroyed and mitotic cells is the criterion employed.

Group C — Colchicine + 500 r. In this group the appearance of the tissue alteration very closely parallels that of irradiation alone. At the highest temperatures, mitotic figures are very rare. However, at 4.5°C a considerable percentage of mitosis is observed.

Control group — no treatment except changes in temperature. The average inherent destruction as observed in 38 tadpoles exposed to temperatures from 0.5°C to approximately 20°C is 0.9%. The number of mitotic figures is also less than 1.0% within these temperatures.

Mitosis vs. 10,000 r and 20,000 r. Thus far only the relationship between mitosis and the damage produced by 500 r hard x-rays has been considered. Figure 5 offers evidence that at the high dosages of 10,000 r and 20,000 r this direct relationship no longer exists.

X-ray resistance in prophase? Since much emphasis has been placed by other workers on radio-sensitive and radio-resistant phases in the cellular cycle, a side experiment was designed to determine whether or not 500 r x-irradiation would cause the destruction of cells which were being arrested in a condition of prophase by colchicine. Three groups of experimental animals were employed:

Group A. Colchicine 24 hours prior to 500 r x-irradiation tadpoles killed 24 hours after irradiation.

Group B. Colchicine 48 hours, tadpoles killed at the end of this time.

Group C. Colchicine 24 hours, tadpoles killed at the end of this time.

All tadpoles were maintained at 20°C.

Examination of the kidney in the three groups revealed:

Group A — 0.9% mitotic figures
Group B — 19.4% mitotic figures
Group C — 9.6% mitotic figures

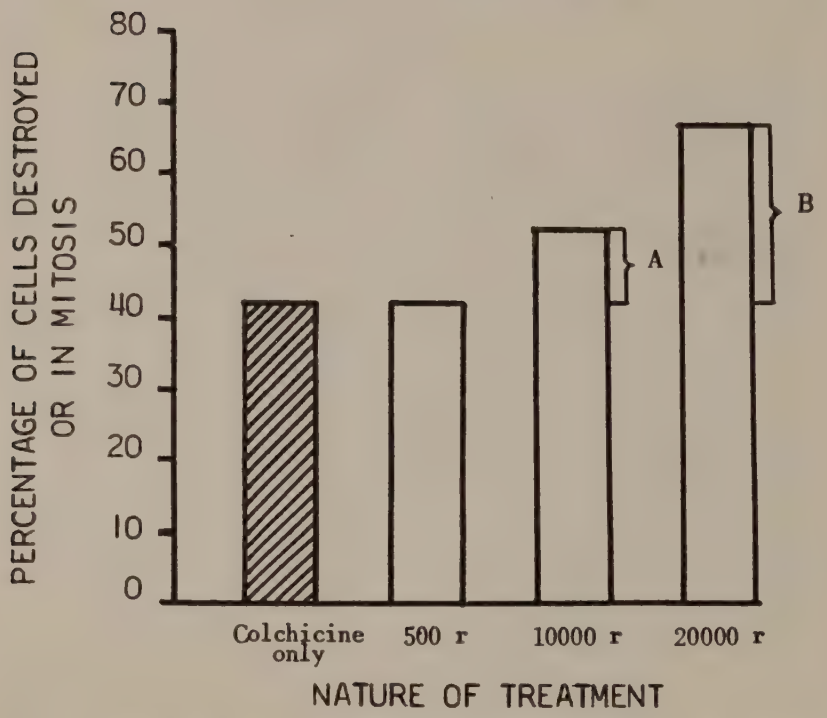

Fig. 5 Relationship between per cent of mitosis and damage by x-rays exceeding 500 r. Increments A (10,000 r) and B (20,000 r) are interpreted as representing percentages of cells which are destroyed independently of cell division as identified by colchicine.

We have already stated that when colchicine and x-rays are administered simultaneously only the irradiation picture prevails. Such simultaneous treatment actually exists during the last 24 hours prior to the killing of group A. Thus one must subtract the number of mitotic figures in group C, from that in group B to obtain an index of the number of mitotic figures which one should observe in group A. This is seen to be

9.8%. It is shown, however, that only 0.9% mitotic figures are observed in group A. Therefore, we conclude that the remaining 8.9% mitotic figures which we would expect to see in this group (A) were destroyed by x-rays. Of course, we emphasize only the qualitative aspects of this experiment.

DISCUSSION

Indications that the sensitivity of cells to ionizing radiation is proportional to mitotic proliferation are abundant in biological literature. As early as 1906, the French workers, Bergonie and Tribondeau ('06) formulated a "law" which reads: "Immature cells and cells in an active stage of division are more sensitive to radiation than are cells which have already acquired their adult morphological and physiological characters."

The special vulnerability to irradiation of certain fast growing tumors, as compared to the normal tissues, appears to be a pertinent illustration. Also supporting this view is the fact that blood-forming tissue, a most active, proliferating tissue, is especially sensitive to irradiation (Dunlap, '42). In 1922, Watanabe and Harrado ('22) demonstrated that increased mitotic activity in the hematopoietic tissue is correlated with increased susceptability to x-rays. These workers stimulated hematopoiesis in rabbits by extensive bleeding some days prior to irradiation and obtained greater destruction of blood-forming tissues by this technique.

Schrek ('46) illustrated the same idea in reverse when he found that neutrophils, which were non-dividing in vitro, were quite resistant to destruction by x-rays. On the other hand, Osgood ('50), who achieved mitosis of these cells in vitro, found them to be very sensitive. Many other observations support this view.

Opposition has more recently appeared to the above long accepted view. Jacobson et al. ('48) found an apparent contradiction when they stimulated hematopoiesis in rabbits some days previous to a test dosage of 800 r, by x-irradiation, bleeding, or administration of acetyl phenylhydrazine. In this

experiment rabbits previously stimulated to increased hematopoiesis by any of these three methods, suffered less injury to the proliferating centers than rabbits which were not so stimulated. Marshak offers evidence that rapidly growing lymphatic tumors are more resistant to irradiation than normal lymphatic precursors.

Thus, the probability that other factors may outweigh mitosis in some instances, and, indeed, that mitosis may not be such a critical stage in all cells, must not be overlooked.

Moreover, even in those instances above which seem to relate x-ray sensitivity to mitosis, there has been no attempt to make quantitative evaluations. For example, although an increase in sensitivity may be generally correlated with rate of mitosis in these tissues, little evidence exists as to whether all the cells being considered are obeying this rule. Also, it is not clear if the cells affected by irradiation are dividing during or after the exposure. We have long believed that the colchicine approach could be effectively utilized in this problem. In our opinion adequate doses of colchicine administered to groups of control tadpoles will establish a relatively accurate picture of cell division in these animals at any given time or period of time which can be quantitatively compared to amounts of x-ray destruction in experimental animals. Osgood ('50) has successfully employed colchicine as a check on the total amount of mitosis occurring in tissue cultures.

Using this technique, we find that the tadpole hematopoietic tissue appears to be susceptible to 500 r in direct correlation with the amount of cell division allowed to proceed following the irradiation period. Cells in prophase at the time 500 r x-irradiation is applied are destroyed directly.

Additional concepts. Thus far we have treated only the general theory that x-ray destruction of cells is quantitatively dependent upon the mitosis following irradiation. If we interpret our findings as evidence for this view, we may also regard them as evidence for concepts in extension to it.

A concept which follows directly is that a condition of mitosis is apparently not necessary for a cell to sustain the initial damage by 500 r. Indeed, colchicine injections show

us that in the tadpole hematopoietic tissue less than 1.0% of cells are in process of division during any one irradiation period.

Our evidence seems to indicate that the primary damage is inflicted in a very high percentage of the hematopoietic cells at the time of irradiation, but this damage is not visible as destruction until these cells begin to divide. Such a point was initially made by Bohn ('03), but since that time has been largely overlooked. In 1941, Spear and Glucksman revived the concept as a result of their findings on the effects of x-rays on the germinative zone in the retina of tadpoles.

It is thus conceivable that some tissues which are not in the ordinary sense considered to be sensitive to x-rays, may actually be just as susceptible to the primary reaction, but may continue for sometime to function normally because of a relatively slow rate of division. However, recovery processes may complicate this simple view.

Halberstaedter and Back ('42) also demonstrated in the colonial protozoan Pandorina that within the dose range of 3,000–300,000 r death was always dependent upon cell division, and Hohl ('49) describes secondary effects in the chromosomes when root cells of plants are irradiated in the interphase.

Our previous work on the tadpole (Allen et al., '50) which demonstrates a delay in manifestation of x-ray damage with a lowering of the tissue temperature, may be regarded as supplementary evidence for this concept. As suggested by Schrek ('46), Patt and Swift ('48), and Duryee ('49), x-ray damage may be divided into two phases. The first phase has a low temperature coefficient accompanied by no visible disturbance, while the second phase has a high temperature coefficient and is correlated with visible damage to the cells.

Mechanisms of x-ray destruction. One may be inclined to ask at this point for chemical-physical explanations regarding the appearance of damage by x-rays at the time of mitosis. For speculations of this nature we must rely heavily on the findings of other workers in the field.

In his recent review Giese ('47) mentions two types of nuclear chemical changes which have been postulated to explain destruction of cells by x-rays. One is the possibility of depolymerization of nucleic acids leading to stickiness and clumping of chromosomes, Darlington ('42). Evidence that ionizing radiations can actually depolymerize nucleic acids in vitro has been offered by Sparrow and Rosenfeld ('46) and Taylor et al. ('47).

The second postulate is that interference with nucleic acid production and conversion may be produced by irradiation. In support of this view Mitchell ('42, 43) has found that radiations stop the formation of thymonucleic acid and ribonucleic acid then accumulates. Mitchell offers calculations to show that a converting enzyme is probably inactivated.

The following paragraphs illustrate how the concept that cells show visible damage from 500 r x-irradiation only upon entering mitosis fits both of the above theories. In fact, both mechanisms and others might conceivably be operating at the same time.

Regarding the first hypothesis, it seems quite possible that nucleic acids or nuclear proteins could be depolymerized in the initial irradiation reaction, Clark ('35). This is a photochemical reaction. As we found in our experiments at low temperatures (Allen et al., '50) there need not be any immediate visible alteration in the cell. But since the nucleic acids are believed to be oriented across the protein fibers of the chromosome, the attempts of a cell to enter mitosis might well result in disorganization of the chromosomes.

In support of the second hypothesis, it is known that deoxyribose nucleoprotein increases in the nuclei of all types of animal cells with a maximum during the prophase, Mirsky ('43). If the enzymes which control this conversion are inactivated in the earlier life of the cell, disruption of the cell can be expected to take place at mitosis.

Mitotic stage vs. sensitivity to x-irradiation. The exact mitotic stage in which sensitivity to irradiation is strongest has been the subject of much dispute. Giese ('47) provides an ex-

cellent table showing the conclusions of 16 different groups of workers who have investigated the problem. Even with the same criteria, differences of opinion still obtain. Each stage has been described by at least one group as the x-ray sensitive one, but 9 of the 16 groups favor the prophase.

Our work offers no indication as to which stage of the mitotic cycle, if any, is most sensitive to x-rays. As before mentioned, we believe that with application of 500 r a large proportion of the hematopoietic cells are affected immediately (non-visibly), although it appears certain that only a very few cells are in a state of active division at the time of x-irradiation. This much, in addition, is demonstrated by our results; if a cell is held in a mitotic stage (early metaphase) by colchicine, administration of 500 r results in destruction of that cell.

Mitotic stage vs. manifestation of x-ray damage. Another question, possibly closely related to the one above, concerns the exact stage of mitosis which is correlated with the visible manifestation of earlier damage. Two things may be said in this regard: (1) In exeriments in which tadpoles have been injected with colchicine and x-irradiated simultaneously, then kept at 13°C or above, no mitotic figures are seen. This observation would lead one to suspect that disintegration of the cell by x-rays is completed in the pre-mitotic phase, before the appearance of well defined mitotic stages. However, (2) when tadpoles are treated with both agents (or x-rays alone) and are then maintained for 24 hours at 4.5°C, mitotic figures in the prophase may be seen.

There is reason to believe that low temperatures provide a "slow motion" picture of cellular events. Thus in these experiments when we see cells in prophase at lower temperatures but none at higher temperatures, we are inclined to believe that lower temperatures provide the more sensitive criteria. The presence of mitotic cells in prophase only, at low temperatures, leads us to conclude that at least some cells irradiated in interphase proceed to prophase before disrupting.

Spear and Glucksmann ('41), who worked on the germinative zone of the retina in tadpoles, also concluded that secondary damage becomes manifest during prophase.

Inhibition of mitosis by x-rays. Inhibition of mitosis as a result of x-rays has been a most common observation and is universally accepted as a measure of x-ray effect. Giese ('47) has presented an extensive review of this subject.

As regards the tadpole hematopoietic tissue, however, such inhibition is difficult to demonstrate with 500 r x-rays. If cell destruction is truly linked to cell division as is implied by the results of our experiments, then examination of the percentage destruction at 2, 4, 8, etc., hours after x-ray should provide an indication of mitotic inhibition. Such examination reveals a linear rather than a wave-like rate of cell destruction from 0 to 15 hours. Allen et al. ('50). This indicates that inhibition of mitosis is either completely overshadowed by destructive processes (more intense than those which would cause mere inhibition) or is not of long duration in the majority of these cells. This does not oppose the findings of Osgood ('50) who obtained marked inhibition of mitosis by 400 r in vitro cultures of immature granulocytes.

Validity of colchicine effects. One of the important assumptions which we have to make for an interpretation of our results is that colchicine, as administered in the dosages employed by us, arrests all hematopoietic cells which attempt division during the experimental period.

In providing an experimental foundation for this assumption, we were confronted with two major problems: (1) A sufficient initial dosage had to be given to stop all cells under observation from completing division. And (2) this effect had to persist over the entire experimental period.

From the experiments and calculations presented by Goldberg et al. ('50) one reaches the conclusion that 3 γ colchicine per gram body weight, which is the L.D.$_{50}$ dosage as far as toxicity is concerned, is the optimum dosage for complete arrest of mitotic cells in frogs. Since the tadpoles used in the present work have averaged 10–15 gm and the injections

of colchicine have been 10 γ to 20 γ per tadpole, these dosages approximate the calculated optimum. Furthermore, we believe we have exceeded the optimum dosage by virtue of the route of injection. Colchicine injected into the base of the tail is carried by the renal portal veins directly to the hematopoietic masses of the kidney. We have unpublished evidence which indicates that this is, indeed, superior to the intraperitoneal and oral routes.

To insure adequate dosage over a long period we used a technique of tail injection in which a prominent pocket of colchicine solution is achieved. This pocket can still be identified at the end of the experiment although reduced in size by constant absorption.

We failed in an extensive search for mitotic figures other than those of the early metaphase, to find any histological evidence that these cells were able to surmount colchicine arrest.

Finally we wish to emphasize our finding of high correlation between percentage of destruction by 500 r x-rays and percentage inhibition of mitosis by both 10 γ and 20 γ colchicine (fig. 1). Such a remarkable correlation becomes difficult to explain if we assume that colchicine does not arrest all cells which attempt to divide.

SUMMARY

1. Cell destruction in a given period following 500 r x-irradiation is directly correlated with rate of cell division during this same time, as determined by colchicine, in tadpole hematopoietic tissue.

2. Rate of mitosis in these cells, as well as rate of cell destruction by 500 r x-irradiation is proportional to temperature.

3. At 10,000 r and 20,000 r the amount of hematopoietic cell destruction in a given period following x-irradiation exceeds cell division during this time.

4. Hematopoietic cells of the tadpole appear to sustain initial invisible damage by 500 r x-irradiation during the rest-

ing state and perhaps all cellular phases. The visible cell destruction is, however, apparently expressed as a secondary characteristic of the initial damage and seems to appear only at the time of cell division.

5. The mitotic phase at which primary indications of damage occur in these cells is probably the prophase.

6. Hematopoietic cells held in the late prophase by colchicine are destroyed by 500 r x-irradiation.

7. Ten gama colchicine via the tail injection route will produce more mitotic figures and less cell destruction than 20 γ injected by the same route. However, the sum of the mitotic figures and destroyed cells is nearly the same in each case.

LITERATURE CITED

ALLEN, B. M., O. A. SCHJEIDE AND L. B. HOCHWALD 1950 Relation of low temperatures to x-ray injury of Hematopoietic tissue of tadpoles. Soc. Exp. Biol. and Med., *73:* 60–62.

———— (Companion Publication.)

BERGNER, D. A. 1950 Studies on colchicine derivatives. III. Effect on mitotic activity of mouse spermatogonia. Cancer, *3:* 134–141.

BERGONIE, J, AND L. TRIBONDEAU 1906 Interpretation de quelques resultat de la radio-therapie et essai de fixation d'une technique rationelle. Cir. Acad. Sci., *142:* 983–985, Paris.

BOHN, C. R. 1903 Influence des rayons du radium sur les aeufs vierges et feconde's et sur les premiers stades du developement. Acad. Sci., *136:* 1085–1086.

CLARK, J. H. 1935 The denaturation of egg albumin by ultra-violet radiation. J. Gen. Physiol., *19:* 199–210.

DARLINGTON, C. D. 1942 Chromosome chemistry and gene action. Nature, *149:* 66–69, London.

DUNLAP, C. E. 1942 Effects of radiation on the blood and the hematopoietic tissues, including the spleen, the thymus, and the lymph nodes. Arch. Path., *34:* 562–608.

DURYEE, W. R. 1949 The nature of radiation injury to amphibian cell nuclei. J. Nat. Cancer Inst., *10:* 735–796.

GIESE, A. C. 1947 Radiations and cell division. The Quarterly Review of Biology, *22:* 253–282.

GOLDBERG, B., L. G. ORTEGA, A. GOLDIN, G. E. ULLYOT AND E. B. SCHOENBACH 1950 Studies on colchicine derivatives. I. Toxicity in mice and effects on mouse Sarcoma 180. Cancer, *3:* 124–129.

HALBERSTAEDTER, L., AND L. BACK 1942 The effect of x-rays on single colonies of pandorina. Brit. J. Radiol., *15:* 124–128.

HOHL, K. 1949 Experimentelle Untersuchungen uber Rontgens Effecte und Chemische Effekte Aus die Pflanzliche Mitose. Georg Thiem Verlag, Stuttgart.

JACOBSON, L. O., E. K. MARKS, E. O. GASTON, E. L. SIMMONS AND M. H. BLOCK 1948 Studies on Radiosensitivity of cells. Science, *107:* 248–250.

MARSHAK, A. 1942 Effects of x-rays and neutrons on mouse lymphoma chromosomes in different stages of the nuclear cycle. Radiology, *39:* 621–626.

MIRSKY, A. E. 1943 Chromosomes and neucleoproteins. Advances in Enzymeology. *3:* 1–34.

MITCHELL, J. S. 1942 Disturbance of nucleic acid metabolism produced by therapeutic doses of x and gamma radiations. II. Accumulation of pentosenucleotides in the cytoplasm. Brit. J. Exp. Path., *23:* 296–309.

———— 1942 III. Inhibition of synthesis of thymonucleic acids by radiations. Brit. J. Exp. Path., *23:* 309–313.

———— 1943 Metabolic effects of therapeutic doses of x and gamma radiations. Brit. J. Exp. Path., *23:* 309–313.

OSGOOD, E. E., AND I. T. CHU 1950 The effect of nitrogen mustard on granulocytic cells as observed by the Marrow Culture Technique. Cancer Research, *10:* 98–102.

PATT, H. M., AND M. N. SWIFT 1948 Influence of temperature on the response of frogs to x-irradiation. Am. J. Physiol., *155:* 388–393.

SCHREK, R. 1946 Studies in vitro on the physiology of cells. Factors affecting the delayed cytocidal action of x-rays. J. Cell. and Comp. Physiol., *28:* 277–304.

SPARROW, A. H., AND F. M. ROSENFELD 1946 X-ray induced depolymerization of thymonucleohistone and of sodium thymonucleate. Science, *104:* 245–246.

SPEAR, F. G., AND A. GLUCKSMANN 1941 The effect of gamma radiation in cells in vivo. III. Spaced radiation. Brit. J. Radiol., *14:* 65–76.

TAYLOR, B., J. P. GREENSTEIN AND A. HOLLAENDER 1947 Effects of x-irradiation on thymus nucleic acid. Science, *105:* 263–264.

WATANABE, M., AND Y. HARRADO 1922 The influence of roentgen rays in hemopoiesis. Jap. Med. World, *2:* 234.

THE INFLUENCE OF TEMPERATURE UPON THE DESTRUCTION OF HEMATOPOIETIC CELLS OF TADPOLES BY X-IRRADIATION[1]

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FOUR FIGURES

There is great need for more quantitative experimental studies upon the factors that condition the susceptibility of tissues to destruction by irradiation.

While much is known about this field of research, we still know too little regarding the partial survival of tissues and their regeneration. We are by no means clear regarding the manner in which irradiation exerts its destructive effects. We have some information upon the relation to it of mitosis and certain biochemical factors but many of the views along these lines are largely hypothetical or rest upon slender factual evidence. The destructive effects of irradiation are probably conditioned by many factors. It is conceivable that some of these may be useful for purposes of control to such a degree as to give a measure of protection at least up to certain levels of exposure. This may, however, prove to be merely a delaying action instead of prevention. We do not know whether lasting modifications of irradiation effects can be achieved.

This, together with the companion paper that follows, introduces a rather ambitious program of study upon the hematopoietic cells of tadpoles. It has long been known that this

[1] This paper is based on work performed under Contract No. AT-04-1-GEN-12. between the Atomic Energy Commission and the University of California at Los Angeles.

tissue is highly susceptible to irradiation, but why is this true? Perhaps it can be explained solely by the active proliferation, as the paper following this appears to show; but there could be other factors as well, perhaps those conditioning mitosis, possibly independent of it. Tadpoles offer many advantages for study along these lines as we shall explain in the section on Materials and Methods.

MATERIALS AND METHODS

While the work embodied in our preliminary paper (Allen et al., '50) showed a uniformly low rate of destruction of hematopoietic cells of x-irradiated tadpoles kept at 0°–5.5°, we were surprised to find in our later work that this is also true at temperatures up through 11°. On the other hand even slight differences in temperature above 11° have a marked effect. Average daily temperatures gleaned from official weather records, sufficed for the conclusions made in our preliminary paper, but they are not adequate for working out a temperature curve such as we present in figure 2, even though our aquarium jars kept in stone tanks shaded by a canopy were found to respond slowly to diurnal temperature fluctuations. This later work at higher temperatures was made possible by adding to our equipment a sensitive and capacious constant temperature water bath.

Obviously work of this kind can be carried out successfully only on cold blooded animals capable of withstanding an extensive range of temperature changes, so for this reason we used bullfrog tadpoles (Rana catesbiana) ideal for the purpose because they can live for days at temperature levels from 0° to 33.5°, which represent the temperature of the organism itself, not merely that of the environment. This species is especially valuable because two years are required to complete metamorphosis, hence it is available the year around.

In tadpoles, the hematopoietic tissue of the kidneys fills the spaces between the renal tubules. While present throughout the entire extent of the kidneys they are most plentiful

in the caudal third and a great bulk of them lie toward the coelomic (ventral) surface. A most satisfactory portion for study is the lateral margin where there is a concentration of hematopoietic cells with little renal tissue.

X-ray treatment was given with a 250 KVP Picker unit. The x-ray factors were 35 cm T.O.D., 15 ma, 0.21 mm Cu inherent + 0.5 mm parabolic Cu + 1.0 mm Al Filters, H.V.L. = 2.1 mm Cu. Roentgens measured in air. Tadpoles were placed in water during irradiation at a deth of 7.0 mm leaving the dorsal half above the surface.

The standard dosage used in these experiments is 500 r requiring about 6 minutes. Before exposure the tadpoles are maintained for several hours at the temperature that is to be employed, while immediately after exposure they are placed in battery jars with water at the same temperature and kept in the constant temperature bath. They are killed at 24 hours after exposure, the kidneys and spleen being fixed in Bouin's fluid. Sections are cut at 5 μ and stained with blood stains — Wright's, Leishman's or Giemsa's. Counts are made with a Whipple grill, 4 typical fields under oil immersion being used. It is quite easy to distinguish the hematopoietic cells which are rather selectively stained. In nearly all cases masses can be found large enough to occupy the entire field of the grill, while in the rare cases where this is not true, partial fields are pieced together in counting. In any case we are dealing with proportions, so errors from this cause are negligible.

The renal tissue shows an extraordinary resistance to x-rays, remaining intact under irradiation of 10,000 r or even 20,000 r, while the adjacent hematopoietic tissue shows extreme destruction. It must be remembered that the hematopoietic cells are in process of very rapid multiplication, as clearly revealed by use of colchicine that arrests mitosis in the early metaphase stage, and also by the presence of large numbers of nuclei in the form of mulberry-like bunches of post-telophase chromosomal vesicles. On the other hand there

are very few mitotic figures in the walls of the renal tubules. We hope to take up these cytological problems in a later paper.

The degenerating nuclei are either in the form of large pycnotic masses or of clusters of degenerating chromosomes or chromosomal vesicles. The frequent dispersion of these causes difficulties in counting, especially in the cases where we are dealing with a group of degenerating cells. However, by being consistent in applying our criteria we feel that our counts are reasonably accurate.

These methods of study give quantitative results that are quite dependable in spite of the high degree of individual variation in susceptibility to x-rays, use of sufficient numbers of specimens in each group giving consistent results.

We have already published a preliminary account (Allen et al., '50) of the effects of low temperatures in this work, which is presented here in the form of a graph (fig. 1) instead of the table used in that article. For details the reader is referred to the preliminary account, however, we might here indicate certain essential points shown in this graph. In the first place the process of cell breakdown is greatly reduced by maintenance of low temperatures following irradiation. Tadpoles chilled before and during irradiation but *subsequently* kept for 24 hours at higher temperatures show almost as high a percentage of destruction as do those maintained at these higher temperatures while being irradiated. On the other hand tadpoles irradiated at a high temperature and immediately afterwards kept at a low temperature show a low percentage of destruction. In short the temperature at the time of irradiation has little if any influence upon the initial (latent) injury, but the temperature after irradiation does control the percentage of cell destruction that develops after irradiation. The current paper throws some light upon the question whether this is merely delay or actual prevention. It adds a great deal to the data presented in the preliminary account regarding temperatures from 0°–5.5° and covers 10 temperature levels above 5.5°. It also extends our studies to time periods above 24 hours up to 10 days.

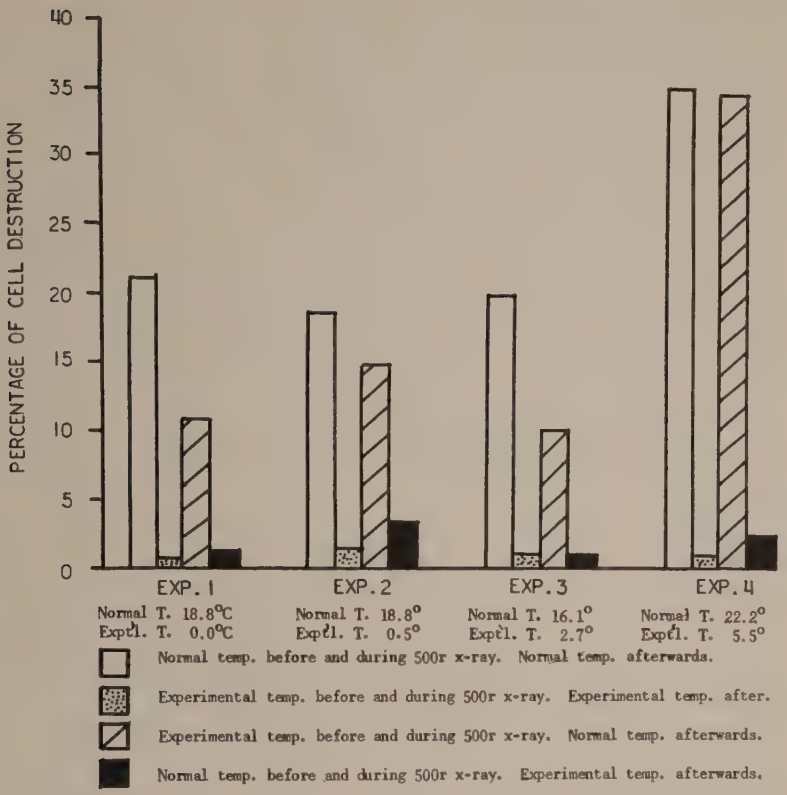

Fig. 1 The percentage of destruction of the hematopoietic cells in the kidneys of tadpoles of Rana catesbiana under different temperature conditions. All killed at the end of 24 hours after irradiation.

RESULTS

Figure 2 shows the sum of our work on the influence of different post-irradiation temperatures upon the destruction of hematopoietic tissue. Each dot represents the percentage of destroyed cells counted in a tadpole, there being 248 altogether. Unirradiated controls were run and counts made in 38 specimens showing an average of apparently dead cells amounting to 0.9%. Ten of these controls chilled 4 days at 2° showed 0.7% of apparently dead cells. There is much doubt as to whether there was even this much cell death because certain normal cells such as red blood corpuscles in small

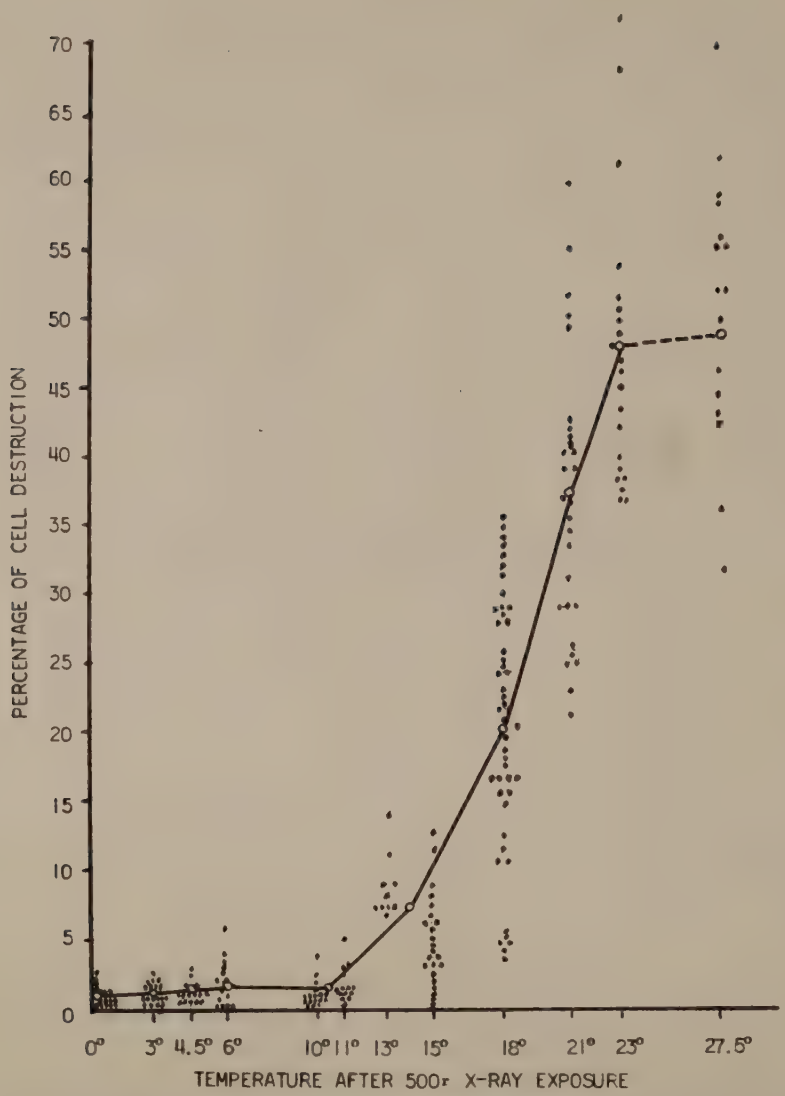

Fig. 2 Graph showing the percentage of destruction of the hematopoietic cells of 248 tadpoles of Rana catesbiana maintained at indicated temperatures for 24 hours after 500 r x-ray exposure. Each dot indicates percentage of destruction in one tadpole. Arrangement of dots is along vertical lines at abscissae except where crowding necessitates spreading. The curve joins circles which indicate averages.

capillaries can be cut in such a way that the compact nuclei simulate those of dead cells. All experimental tadpoles were subjected to 500 r x-ray dosage and killed at the end of 24 hours after being kept at the given temperatures throughout that time. The dots, arranged in vertical lines indicate a considerable degree of variation and yet in places they are so bunched as to require being placed side by side in clusters. The high degree of variation is in line with the findings regarding susceptibility to x-rays in general. In this graph the average of tadpoles maintained at a given temperature is indicated by a circle.

While our earlier work had shown that post-irradiation refrigerator temperatures of 0°–5.5° greatly delayed the breakdown of x-irradiated hematopoietic tissue we were surprised to find that equal retardation is produced by temperatures of 10° and 11°. Beginning to rise at 13° and 15°, the percentage of destruction increases very rapidly at 18°. This critical temperature shows a very great degree of individual variation probably because of its threshold position.

At 23° the apparent destruction has almost reached a climax, 27.5° showing a percentage only slightly greater. At the higher temperatures there is probably a large amount of resorption within the 24 hours, which may account for the bending of the curve between 23° and 27.5°. While there may be some of this resorption at the lower temperatures, there is a vary great amount of it at 33.5°, so that to include in the graph the results at this temperature would be highly misleading. The evident diminution of the hematopoietic tissue observed at the end of 24 hours at this temperature induced us to run a special series at shorter intervals. There were 8 stages of 10 or more tadpoles each, killed at intervals ranging from two to 24 hours. Ten per cent were found to be destroyed in the lot killed at the end of two hours and the maximum percentage of destroyed cells was observed in the lot killed at 15 hours. Since we are dealing with the percentage of dead cells observed at each of these time intervals (fig. 3), we can only infer the details of their resorption, but the small number

left at the end of 24 hours is clear evidence of their rapid degeneration and disappearance when we take into account the very high percentage of dead cells observed at 12 and 15 hours.

On the other hand, counts of dead cells at the lower temperatures (4.5° and 10°) show a consistent percentage of slow increase up to 10 days after x-ray treatment (fig. 3). We do not claim that there is a complete lack of resorption of dead cells during the 10 days at these low temperatures, but

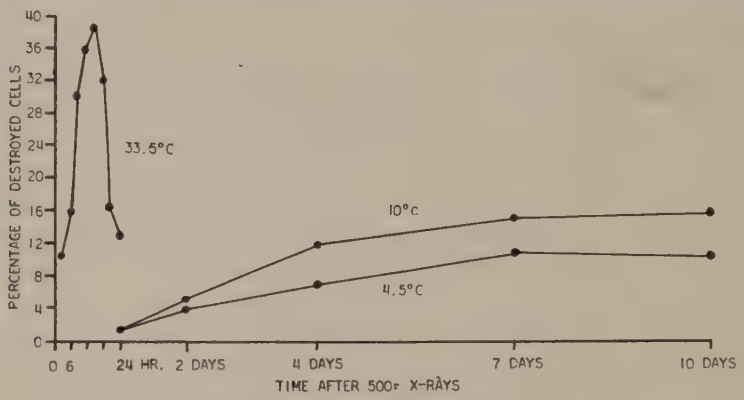

Fig. 3 Percentage of destruction of the hematopoietic cells of Rana catesbiana subjected to different temperatures after x-ray exposure of 500 r. Each curve represents tadpoles maintained at indicated temperature. Groups of 10 are indicated by each dot.

the total number of living and dead cells remaining at the end of this period shows that there must be very little if any. It is clear that these graphs, figures 2 and 3, do not show absolute values especially at higher temperatures because they represent percentages based upon counts of cells living and dead in tissues killed at the end of 24 hours, except the 33.5° graph of figure 3. Destroyed cells that have been resorbed prior to fixation of the tissues have disappeared without entering into the counts. Additional work might give us data upon which the absolute values could be estimated but this would scarcely be worth the trouble. The main principles

involved are clear enough and easily deduced from the graphs as they stand.

Figure 4 shows experiments upon two lots x-irradiated together and maintained side by side at 4.5° in the refrigerator. At the end of 10 days one lot is killed and the other transferred to a temperature of 18° for 24 hours additional. This lot is then killed and the percentage of destroyed cells is found to be a component of the two temperature exposures. The broken line at the left shows the percentage of destruction in 24 hours at 18° as previously determined (fig. 2).

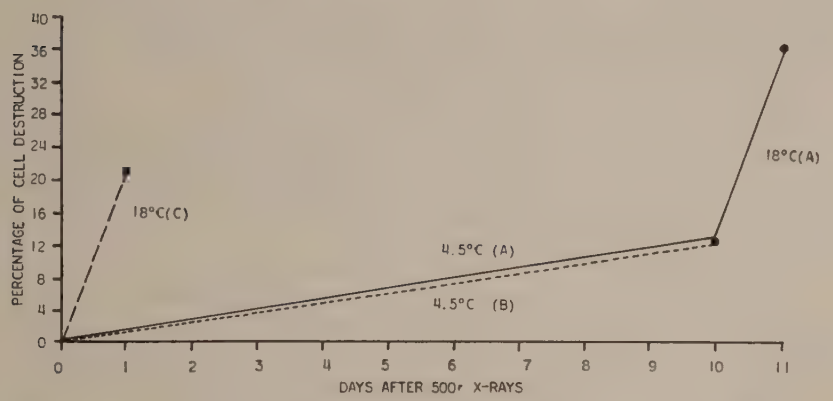

Fig. 4 Showing additive effect of changed temperature level on destruction of hematopoietic cells produced by 500 r x-ray exposure of tadpoles of Rana catesbiana. Three lots of 10 each: (A) Kept at 4.5° for 10 days, then raised to a temperature of 18° for one day additional. (B) Kept at 4.5° for 10 days. (C) Kept at 18° for one day.

DISCUSSION

At the beginning of our work we determined the $L.D._{50}$ of this species of tadpoles finding it to be 10,000 r on a basis of 35 days. Of course, this dosage is fantastically high in comparison with those representing the $L.D._{50}$ for mammals, but at the same time the destruction of hematopoietic cells, forming the object of this work is very extensive at 500 r, which is near the $L.D._{50}$ for many mammals. It is clear that the $L.D._{50}$ determination is of little value in this work on tadpoles because it is not an index of the destructive effect of x-ray

treatment on the hematopoietic cells but indicates rather the susceptibility of the organism as a whole which is not so responsive to destruction of the hematopoietic cells in the tadpole as it is in the mammals. Our choice of 500 r of hard x-rays as a standard dosage was a practical one but the destruction that it causes in tadpoles may well bear comparison with that produced in, say, the rat.

We have not taken the pH into account because we have been careful to equalize conditions for experimental and control tadpoles. In fact, the tadpoles have surprising ability to regulate the pH of the water in which they are kept, as we found in some preliminary tests be placing them in jars of water with pH strongly acid or alkaline.

This work shows that the post-irradiation temperature factor is very important. No irradiation experiments with cold blooded animals can have quantitative value unless a known constant temperature is accurately maintained during the period following exposure.

Among those who have shown the importance of the temperatures at which organisms are maintained following irradiation, are Packard ('30) with Drosophila eggs; Tahmisian ('49) with eggs of the grasshopper — Melanoplus; Spear and Glücksmann ('28) with the proliferating cells of the retina of tadpoles; Duryee ('49) with salamander oöcytes; and Strangeway, Fell and Fell ('27, '28) with chick embryos.

The opposite view, namely that cold increases susceptibility to irradiation, has been advanced by Crabtree and Cramer ('33) with transplantation of chilled slices of mouse cancer tissue and by Mottram ('35) with root tips of the board bean. In the Crabtree and Cramer ('33) work only the normal temperature of the host mice was involved and Mottram gives no data regarding the temperature following irradiation.

Some of the earlier workers in this field claimed that low temperatures do not have any restraining effect on radiation damage; but their experiments dealt only with the temperature at the time of irradiation, not with that at which they

were subsequently kept. Examples of this type of experiment are Henshaw and Frances ('33) with wheat seedlings; Ancel and Vintemburger ('27) with eggs of the frog and hen; and Ancel ('27) with bean sprouts. These results are in line with our own in so far as they deal with experimental temperatures applied only at the time of irradiation but not maintained subsequent to it.

Newborn rats can tolerate quite low temperatures and have been used by Evans ('41) and co-workers for the study of this problem. The skin was irradiated at 0° and 26° and it was found that destruction at the higher temperature was more severe than at the lower. Here we are dealing with the influence of temperature at the time of irradiation with apparently no control exerted afterwards. This work appears to be at variance with our own and that of other workers as discussed above. It is hoped that experiments on new born rats might be devised to test the effects of different temperatures after irradiation.

The work of Schrek ('46) upon treatment of mammalian tissues in vitro is important in this field, especially the part dealing with irradiation of cultures of the thymus cells of rabbits. He clearly distinguishes between cases in which the experimental temperature is applied only during irradiation and those in which it is applied afterwards during incubation showing that temperature is effective only in the latter case. His work is especially interesting in that he locates at 17° the critical temperature which marks the point in his graph where the destruction shows a sharp increase. His temperature intervals were 2°, 5°, 7°, 17°, 27°, etc., so this point is not a narrowly defined one. In our own work the critical rise in destruction started at 13°, ascending sharply at 18° and continuing to rise at higher temperatures. Thus his results and our own are in general correspondence. This point is quite interesting in that there is involved a comparison between a cold blooded and a warm blooded animal, also between in vitro and in vivo treatment.

One of the very significant papers bearing upon our work is that of Patt and Swift ('48), in which they show that adult frogs (Rana pipiens) irradiated at 1,000 r, 3,000 r, 6,000 r and 9,000 r die within two weeks if kept at a temperature of 23°, but when kept at 5°–6°, life is prolonged for at least 60 days with a very low percentage of mortality varying somewhat in direct proportion to the x-ray dosage. They also find that refrigeration during irradiation, and for only 24 hours afterward, does not materially prolong life. It is also found that x-irradiated frogs kept alive by refrigeration for weeks die after the characteristic interval of time following transfer to a temperature of 23°.

Our problem differs in several ways from theirs in that we are working upon the hematopoietic cells alone in larval forms of a different species and using different criteria of measurement, yet the parallelism appears to be quite significant.

In this brief survey of literature it is seen that our results show substantial correspondence with those of a considerable number of others in the field.

In our companion paper, "The Relation of Mitosis to the Manifestation of X-ray Damage in the Hematopoietic Cells of Tadpoles," to which the reader is referred, we have shown the close association of mitosis with x-ray destruction. Furthermore we have under way a number of experiments upon other factors affecting x-ray damage.

SUMMARY

1. Post-irradiation temperature controls the rate of destruction of the hematopoietic tissue as shown by the use of 13 different temperatures ranging from 0°–33.5°, illustrated in figure 2.

2. The breakdown of x-irradiated hematopoietic cells is very slight at post-irradiation temperatures below 13° but increases quite rapidly in the 10 degrees between 13° and 23°, continuing to rise in intensity to the highest temperature tolerated.

3. The rate of destruction is conditioned only by the temperature at which the tadpoles are kept *after* exposure to x-rays, slight apparent effects of chilling before and during x-ray treatment probably being due to delay of the tissues in reaching the given post-irradiation temperature level of the experiment, and in regaining a normal physiological condition.

4. If a low temperature applied for several days is changed to a higher temperature, the destruction of hematopoietic cells changes to the level characteristic of the higher temperature, the total destruction being the sum of the two. This suggests that there is little if any recovery during long retention at these low temperatures, which have apparently operated only to delay destruction.

5. Irradiation produces injuries that may remain invisible for some time, awaiting reactions or changes in the cells. Degeneration of the cells is closely associated with mitosis. Evidence along this line is presented in our companion paper which shows the close correspondence between the rate of mitosis and the rate of destruction ascending together at higher temperature levels.

LITERATURE CITED

Allen, Bennet M., Ole Arne Schjeide and Lynette B. Hochwald 1950 Relation of low temperatures to x-ray injury of hematopoietic tissue of tadpoles. Proc. Soc. Exp. Biol. and Med., *73:* 60–62.

Ancel, Suzanne 1927 Influence de la temperature sur la radio-sensibilité de grains de lentilles au cours de la germination. C. R. Soc. de Biol., *97:* 800–802.

Ancel, P., and P. Vintemburger 1927 Influence de la temperature sur la radiosentibilité d'oefs d'oiseau et de Batrachiens. C. R. Soc. de Biol., *97:* 796–793.

Crabtree, H. G., and W. Cramer 1933 The action of radium on cancer cells. II. Some factors determining the susceptibility of cancer cells to radium. Proc. Roy. Soc., London, S. B. *113:* 238–250.

Duryee, W. R. 1949 The nature of radiation injury to amphibian cell nuclei. J. Nat. Cancer Inst.. *10:* 735–796.

Evans, T. C. 1941 The effects of roentgen irradiation at low temperatures on the skin of young rats. Am. J. Roentgenol., *45:* 888–894.

Henshaw, P. S., and D. S. Francis 1933 Growth rate and sensitivity of Triticum vulgare. J. Cell. and Comp. Physiol., *4:* 111–122.

MOTTRAM, J. C. 1935 On the alteration in the sensitivity of cells toward radiation produced by cold and anaerobiosis. Brit. J. Radiol., *8:* 32–39.

PACKARD, CHARLES. 1930 The relation between division rate and the radiosensitivity of cells. J. Cancer Res., *14:* 359–369.

PATT, HARVEY M., AND MARGUERITE N. SWIFT 1948 Influence of temperature on the response of frogs to x-irradiaton. Am. J. Physiol., *155:* 388–393.

SCHREK, ROBERT 1946 Studies in vitro on the physiology of cells. Factors affecting the delayed cytocidal action of x-rays. J. Cell. and Comp. Physiol., *28:* 277–304.

SPEAR, F. G., AND A. GLÜCKSMANN 1928 The effect of gamma radiation on cells in vivo. I. Brit. J. Radiation, *11:*533–553.

STRANGEWAY, T., S. P. FELL AND H. B. FELL 1927–28 A study of the direct and indirect action of x-rays upon the tissue of embryonic fowl. Proc. Roy. Soc., London, S. B. *102:* 9–29.

TAHMISIAN, THEODORE N. 1949 The effect of x-irradiation on the metabolic process of the resting cell. J. Exp. Zool., *112:* 449–464.

PENETRATION OF RADIOACTIVE ISOTOPES, P^{32}, NA^{24} AND K^{42} INTO NITELLA [1]

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THREE FIGURES

I. Penetration of radioactive phosphate by the continuous method

In resuming the study of the intake of radioactive ions, it seemed desirable to appraise the role of variability in the calculated data either inherent in the living cell of a species of *Nitella,* or due to variations attributable to the technique. The earlier work (Brooks, '38, '39, '41, '43, '45) showed definite rhythm in intake of radioactive ions by isolated cells, and for this reason it was thought of interest to repeat some of these experiments by other methods.

In the previous experiments (ibid.) cells of *Nitella* were immersed in the experimental solution for certain periods of time, and then taken out and measured during the experiment, involving handling. Since *Nitella* is sensitive and easily injured, it was thought important to use a method which enabled measurements to be taken without subjecting the cells to handling. The method used was designated as the "continuous method" and consisted of perfusing the cell after it had been sucked into a capillary tube. In this way the cell was bathed continuously and the fluid changed as desired by turning a stop-cock. In making the readings, the entire capillary was placed over the Geiger Muller counter. Readings were therefore made with the cell intact and bathed by

[1] Aided by a Research Grant, United States Public Health Service. Preliminary Report, Western Society of Naturalists, at Los Angeles, California, December 1947.

either the experimental solution or by distilled water. Controls were also made of the same capillary tube without the *Nitella* cell.

The *Nitella* cell was prepared at least two weeks previously when it was removed from pond water and brought into the laboratory. The cells on either side of the central cell were in good condition as indicated by streaming. All portions of the cell except the internodal portion were shielded from radiations by using thin strips of lead. During the experiment, the cells were observed in the microscope for streaming. All experiments in which streaming became sluggish, or in which degeneration of the Chloroplasts occurred were omitted. The temperature was kept constant by a water jacket at 15°C.

The background was measured with the *Nitella* cell in place. The radioactive solution was then perfused through the capillary tube and continuous measurements were made. At the end of the experiment, the radioactive solution was replaced by pond water and the activity emanating under these conditions was measured. The cell was then removed and the capillary tube again counted for emanations with the radioactive solution alone present. Finally, the intact cell was separated into its component parts, namely, sap, protoplasm and cell wall. These were separately dried and measured for activity.

RESULTS

There were several experiments in this group. They were similar in results. However, only experiment 5 will be fully described here and the complete data for determining the permeability constant as described in Brooks and Brooks ('41), page 84 et seq. computed as follows:

$P = GM \cdot cm^{-2}$ sec.$^{-1}$ $(GM \cdot L^{-1})^{-1}$ in which P is the permeability constant; GM here is represented by counts per minute (c/m). The c/m of the external solution as compared with the c/m of the experimental *Nitella* cell would indicate the concentration gradient between outside and inside the cell as shown in table 1; cm^{-2} is the area of the cell;

L represents the change to liters to conform with the equation; sec. is time in seconds.

The activities are as follows: that of the external solution following the capillary tube and kept constant by flow, without the *Nitella* cell was 850 c/m or 0.0125 μc/ml; that of the *Nitella* cell, 250 c/m.

The pH of the external solution was 6.0.

Explanation of table 1

The equation for permeability as taken from Brooks and Brooks ('41, p. 84) has substitutions of c/m (counts per minute) for GM, representing the concentration or activity of the external solution and/or that of the *Nitella* cell which had accumulated the radioactive phosphate. In these experiments the volume of a section of the cell measuring 41 mm was 0.0153 cm^3. The c/m of a capillary tube of the same length without the cell containing the experimental solution minus c/m of the capillary tube with the cell was $850 - 250 = 600$ c/m. Changing the figures for volume of the section of the cell used to liters, we have $15.3 \times 10^3 \times 600$ which equals 39.2×10^6. The factor in column 5 should be: $\frac{39.2 \times 10^6}{850 \text{ (used above)}} = 4.62 \times 10^4$. Column 6 represents the change from minutes to seconds as specified by the equation.

At two hours the radioactive solution was replaced by distilled water and measurements made for two hours. There was a slight drop in the rate of uptake which remained constant. The drop can be attributed to the washing away of fluid surrounding the *Nitella* cell. The loss period has been computed as follows:

c/m $= \frac{50}{850 \times 120} = 0.00049$. The "P" constant $= .17 \times 10^{-9}$.

Column 1 represents time in minutes; column 2, c/m for the whole cell plus the external solution; column 4, simplifying the calculations by equating the values found to 1.00, square centimeter as area.

In this table the equation as outlined in Brooks and Brooks (ibid.) has been used. Substitutions for GM were c/m.

TABLE 1

Permeability constants for seconds

INTAKE TIME IN MINUTES	C/M FOR WHOLE CELL + EXT. SOL.	$\frac{\Delta \text{ C/M}}{\text{EXT. SOL.} \times \Delta \text{ TIME}} \div$	100 CM² = AREA ÷	FACTOR = 4.62×10^4	"P" IN SEC. $\times 10^{-9}$
1	2	3	4	5	6
				$\times 10^{-7}$	
0	250	$\frac{625}{850 \times 30} = 0.0245$	0.0245	5.30	8.8
2	289				
30	875				
60	1375	$\frac{500}{850 \times 30} = 0.0190$	0.0190	4.11	6.8
90	1775	$\frac{400}{850 \times 30} = 0.0156$	0.0156	3.37	5.6
120	2175	$\frac{400}{850 \times 30} = 0.0156$	0.0156	3.37	5.6
15	625 Δ from 0	$\frac{375}{850 \times 15} = 0.0290$	0.0290	6.28	10.5
15	Δ from 30	$\frac{250}{850 \times 15} = 0.0196$	0.0196	4.25	7.1
		Calculation for loss period in water			
120	50	$\frac{50}{850 \times 120} = 0.00049$	0.00049	.106	.176
		Calculation for loss period in $Na_xH_yPO_4$			
90	125	$\frac{125}{850 \times 90} = 0.00163$	0.00163	.35	.50

$P = GM \cdot cm^{-2} \cdot sec.^{-1} \cdot (GM/L^{-1})^{-1}$, in which P is the permeability constant.

The same equation is used for computing the value of "P" for the loss periods which occurred when the cell was placed for 90 minutes in .0025 M concentration of $Na_xH_yPO_4$.

Figure 1 shows the curves for table 1, representing the rate of penetration of radioactive PO_4 into *Nitella* cells during the course of 6 hours. The abscissae represent time in minutes after the start of perfusion and the ordinates, counts

per minute with the cell in the capillary tube. At 125.5 minutes, perfusion was stopped and the cell was washed with double distilled water. At 254 minutes, the cell was perfused with .0025 M $Na_xH_yPO_4$ buffer at pH 6.0. The graph shows that there is an initially rapid uptake of PO_4 with a gradual slowing down. The addition of distilled water caused a drop due to the removal of excess perfusing fluid, after which the decrease was very slight.

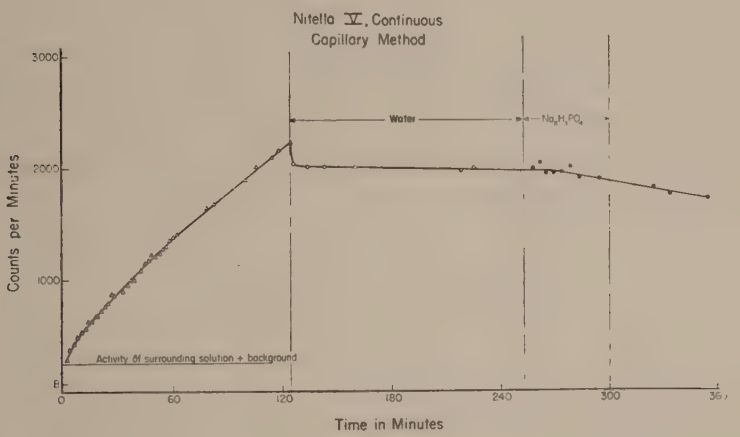

Fig. 1 Shows the rate of penetration of P_{32}* into whole *Nitella* cells. The abscissae represent time in minutes and the ordinates, counts per minute per unit volume as calculated. During the first 120 minutes the cell was perfused with radioactive PO_4; followed by water during the next period and finally by $Na_xH_yPO_4$. The activity of the surrounding solution and background are indicated.

The PO_4* content of the perfusing medium was 1×10^{-11}. During the intake of P^{32} there is also a negligible loss of P^{32} by ionic exchange but which has not been computed here. The external concentration of P^{32} is a constant due to the perfusion method.

At the end of the experiment, the sap, wall and protoplasm were measured separately. The values for these results are found in table 2.

These figures show that the part of the cells which is actively permeable to these ions is the protoplasm.

TABLE 2

PART OF CELL	COUNTS PER MIN./ML
Sap without protoplasm (dried)	0
Cell wall and protoplasm (dried)	3282
Cell wall separately	nil +

CONCLUSION

The protoplasm is the only constituent of the cell into which PO_4^* has penetrated. The cell sap has none after 6 hours, and the wall has either none or a very negligible amount.

The rate of uptake of P_{32}^* is smooth and not rhythmic when the concentration of the surrounding medium is kept constant. This factor is probably responsible for the rhythmic results found in previous work.

II. Penetration of radioactive PO_4 by the discontinuous method

In this group of experiments *Nitella* cells were placed in the experimental solution instead of being perfused. There were 10 determinations for each reading. Pond water was used for dilution. The temperature was kept at 15°C. $NaHPO_4$ was added to give a concentration from 5.0 to 6.5×10^{-4} gm per liter. The pH was kept at either 6.9 or 7.2. Filaments of *Nitella* were trimmed of all side branches at least two weeks before use as in the previous experiments, and allowed to remain in pond water. Only those in good condition, turgid and dark green were used. For each reading 10 filaments of from 4 to 7 cells were used. They were immersed in radioactive PO_4 solution having an activity of .05 μc per milliliter and were kept individually for periods of time from 4 to 30 minutes. Immediately after removal they were washed in distilled water for 15 seconds, the sap expressed from each cell individually and the aggregate volume of sap from each filament determined. The sap, cell wall and protoplasm were separately dried and the activities determined. The activity of the sap and the corresponding cell wall and protoplasm

was computed on the basis of .02 cm^3 throughout the measurements as in the previous experiments (table 1) because a cell which contains .02 cm^3 of sap has a surface area of 1.00 cm^2. Each determination is based on an average of from 40 to 70 cells. The rate of uptake of PO_4 for the first 15 to 30 minutes after immersion was constant. Thereafter the rate slowly decreased.

TABLE 3

Immersion method series

1	2	3	4	5
NO. OF EXP.	TIME IN MIN.	GM/L OF EXTERNAL SOLUTION	GM/CM^2 SEC.	P
4	0–15	5.0×10^{-4}	$.11 \times 10^{-11}$	$.022 \times 10^{-7}$
5	0–30	6.5×10^{-4}	$.18 \times 10^{-11}$	$.028 \times 10^{-7}$
6	0–25	6.5×10^{-4}	$.28 \times 10^{-11}$	$.043 \times 10^{-7}$
7	0–25	6.5×10^{-4}	$.19 \times 10^{-11}$	$.029 \times 10^{-7}$
8	0–20	6.5×10^{-4}	$.6 \times 10^{-11}$	$.092 \times 10^{-7}$

In table 3 are the results of 5 experiments showing the rate of penetration of PO_4* into *Nitella* by the immersion method or the "discontinuous method."

The first column represents the number of the experiment; the second, time in minutes; the third column, the concentration of radioactive PO_4 in the external solution in grams per liter; the 4th, the rate of penetration of PO_4* into *Nitella*

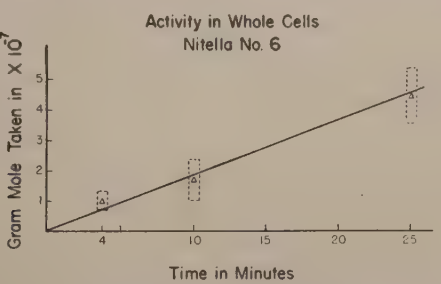

Fig. 2 Shows the rate of penetration of radioactive PO_4 into whole *Nitella* cells by the discontinuous method. The probable error is represented by the rectangles. Abscissae are time in minutes; ordinates, gram moles times 10^{-7} taken in by the plant.

cells of the designated area and time; the 5th column, the permeability constant as figured in table 1 (dividing the figs. in column 4 by those in column 3).

Figure 2 represents one of 8 typical experiments and is derived from experiment 6 (table 3). The abscissae represent grams of PO_4^* entering *Nitella*. The ordinates represent time in minutes. The figure shows that the rate of penetration is constant for at least the first 25 minutes.

CONCLUSIONS

These results show again the steady rate of penetration of radioactive PO_4 into *Nitella* cells and that the values for the permeability constant P, are consistent. Accumulation takes place in the protoplasm with no penetration into the sap. The small amount in the sap which was sometimes found, can be attributed to contamination from the protoplasm.

III. The penetration of Na^{24} and K^{42} into Nitella

In these experiments with Na^{24} and K^{42}, the plants were treated in the same manner as previously described, and only those remaining dark green and turgid used. They were laid in their respective experimental solutions for specified periods of time and taken out of the solution when measured by the Geiger Müller counter. The temperature was kept between 14 and 15°C. The concentration of NaCl including the radioactive amounts added was .0027 M in no. 1 Na; .0625 in no. 3 Na and in the case of no. 1 K, the experimental solution remained "carrier-free," so that no measurements are given.

Table 4 shows the rate of penetration of Na^* in the first two experiments and K^* in the third. In all cases there is considerably greater concentration of radioactive Na or K penetrating the protoplasm than the sap as shown in column 3 as compared with column 4. The counts per minute are given for .02 ml in each case. Column 5 represents the counts per minute of .02 ml of external solution; column 6, the M

concentration of the external solution; column 7, the activity of the external solution and column 8, its pH.

Figure 3 represents the experiment, Na no. 3 from the above table, and gives the separate readings showing the extreme variability of the results. The probable error is shown by the rectangles and the probable uptake of radioactive Na in the protoplasm by the line drawn through the calculated points. Calculations for the sap are also represented and show a low concentration as represented by the

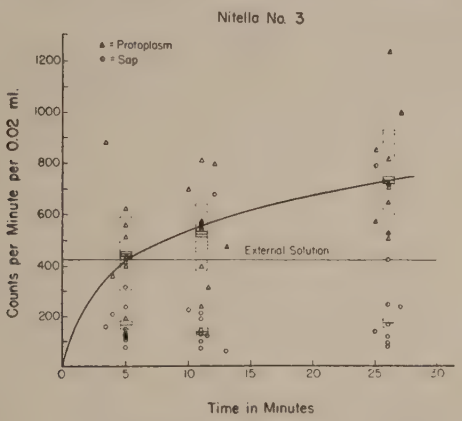

Fig. 3 Represents one experiment in which the penetration of radioactive Na is illustrated. The triangles represent protoplasm and the circles, sap; abscissae, time in minutes; ordinates, counts per minute per unit volume. Rectangles are the limits of probable error. The external solution is shown by a straight line.

squares. The abscissae represent time in minutes and the ordinates, counts per minute for 0.02 ml volume of solution. The concentration of the external solution is indicated.

In this table the background is already subtracted from the total number of counts. The counts are within 3% accuracy.

The curves for the penetration of Na^* and K^* are smooth and exponential in nature. The concentration in the sap in both cases remains considerably below that in the protoplasm. It is probable that a large portion of the activity

of the sap is due to contamination by fragments expressed from the protoplasm on extraction. There is considerable spread in these readings by this method. With the availability of larger supplies of radioactive elements and the opportunity for taking more readings, it is evident that the irregularity shown by the curves in the past (ibid. Brooks) was due to this factor.

TABLE 4

Penetration of Na^{24} and K^{42} into Nitella

1	2	3	4	5	6	7	8
NO. OF EXP.	TIME IN MIN.	PROTOPLASM C/M PER .02 ML CELL VOL.	SAP C/M PER .02 ML	EXT. SOL. C/M PER .02 ML	CONC. OF EXT. SOL. MOLES/L	AC-TIVITY μO/ML	pH
No. 1							
Na^{24}	30	312	69	149	0.0027 M	.05	7.0
	90	326	104	149	0.0027 M	.05	7.0
No. 3							
Na^{24}	5	438 ± 13	157 ± 8.0	429	0.0625	.10	6.4
	11	515 ± 14.1	136 ± 13.2	429	0.0625	.10	6.4
	26	731 ± 21.7	172 ± 5.2	429	0.0625	.10	6.4
No. 1							
K^{42}	4	532	80	212	0.000 ?	.10	6.9
	10	1229	171	212	0.000 ?	.10	6.9
	25	1780	244	128	..	..	6.9

Not sufficient data are given to compute the permeability constant of this set of experiments. But it can be concluded that there is penetration of these radioactive ions into both the sap and the protoplasm, and that accumulation takes place in the protoplasm.

DISCUSSION

From these results, it may be deduced that differences in the results found for the rate of intake of radioactive ions by isolated cells in this case as compared with former experiments, may be considered as due to the methods used.

In previous papers the remarkable variation had not been sufficiently appraised owing to great difficulties in the early

isotope work. In the experiments described in this paper it was possible to make a sufficiently large number of readings to compensate for individual variations. It is also possible that in the earlier work where the individual *Nitella* cells were removed from their external solution and suspended in air during counting, injury may have resulted. It was found that by merely touching a *Nitella* cell with a probe, streaming ceases temporarily, illustrating that the cell is highly sensitive to handling. Furthermore, removal from the solution causes the cells to lose turgor within a minute.

In the continuous perfusion experiments these difficulties were eliminated since the cells were at no time exposed to air nor disturbed. With these newer methods, the rhythms found in the earlier work were eliminated and smooth curves obtained.

The perfusion experiments with *Nitella* and PO_4^* were done with the assistance of E. L. Chambers; the immersion experiments with *Nitella* and Na^{24}, K^{42} and PO_4^*, with the assistance of R. Kutsky. The manuscript was assembled by Matilda M. Brooks with the assistance of E. L. Chambers.

LITERATURE CITED

Brooks, S. C. 1938 J. Cell. and Comp. Physiol., *11:* 247–252.

———— 1939 J. Cell. and Comp. Physiol., *14:* 383–401

———— 1943 Biol. Bull., *84:* 213–225.

———— 1945 Ann. Review Physiology VII, 1–33.

Brooks, S. C., and M. M. Brooks 1941 Gebruder Borntraeger. The Permeability of Living Cells. Berlin.

Mullins, L. J., and S. C. Brooks 1939 Science, *90:* 256.

THE EFFECT OF ANESTHETICS ON CALCIUM RELEASE[1]

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INTRODUCTION

Qualitative evidence indicates that fat solvent anesthetics such as ether and chloroform cause a release of calcium in the cells of the aquatic plant, Elodea (Mazia and Clark, '36), and in the protozoan, *Amoeba proteus* (Daugherty, '37). This study presents some quantitative evidence of calcium release in cells of higher animals under the influence of both fat solvent and non-fat solvent anesthetics (cocaine).

MATERIALS AND METHODS

1. *Anesthetic solutions.* The anesthetic solutions used were 2% ether, 0.4% chloroform and 2% cocaine in unbuffered frog Ringer. According to Winterstein ('26, pp. 13 and 294), these are anesthetic concentrations for frogs.

2. *Muscle fiber preparations.* The combined right and left gastrocnemei of 4 frogs (*Rana pipiens*) were used for each experiment. The whole muscles were removed from each leg and placed in 1 ml of frog Ringer solution on a black glazed plate. The muscle fibers were teased away from the connective tissue with fine steel needles. The fibers from the 4 pairs of muscles were thoroughly mixed, lightly blotted with calcium-

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free filter paper (Whatman No. 42) and divided into 4 approximately equal parts. Each part was then transferred to a watch glass and weighed.

One of the 4 parts was placed in 25 ml of 2% ether-Ringer solution, a second in 25 ml of 0.4% chloroform-Ringer solution, a third in 25 ml of 2% cocaine-Ringer solution, and the 4th in 25 ml of frog Ringer solution (control). After one hour of soaking, the fibers were removed by filtration through calcium-free filter paper (Whatman No. 42). The fibers were washed with 5 ml of distilled water, and the washings added to the filtrate.

Aliquots of each filtrate were removed for the determination of calcium. These determinatons were corrected for the amount of calcium originally present in the frog Ringer solution.

After the filtration, the muscle fibers were placed in 10 ml of isotonic KCl, and subjected to ultrafiltration. The ultrafiltration apparatus and pressure regulator was a modification of that described by Greenberg and Gunther ('30), being arranged so that the 4 groups of fibers could be treated simultaneously. A negative pressure of 150 mm of mercury was applied. The collodion sacs used were made of a solution of 7 parts of collodion, 60 parts of ethyl ether, 40 parts of absolute alcohol, and 5 parts of ethylene glycol (Mazia, '37). Determinations done on the sacs alone showed that they contained no measurable calcium. Twelve to 18 hours at 5°C. was always sufficient for complete filtration. The calcium in 4 ml aliquots of the ultrafiltrates was determined as a measure of the free calcium in the fibers (Greenberg and Gunther, '30). The fibers themselves were transferred to Folin-Wu digestion tubes and ashed with 5 ml of a 1:1 mixture of concentrated sulfuric and concentrated nitric acids per gram wet weight of fibers. When the ashing was completed, the solutions were made to 35 ml with distilled water. The calcium in 4 ml aliquots of these final solutions was determined as a measure of the bound calcium in the fibers (Greenberg and Gunther, '30). For each experiment, then,

there were three determinations of calcium for each of 4 conditions: (1) in the soaking solution; (2) in the ultrafiltrate, and (3) in the residue from the ultrafiltration. The 4 conditions were: (1) Ringer (control); (2) ether; (3) cocaine; and (4) chloroform.

3. *Calcium determinations.* Calcium was determined after the method of Larson and Greenberg ('38). Aliquots of the solutions to be analyzed were adjusted to pH 5.4, using 28% ammonium hydroxide with brom-cresol green as the indicator. The calcium in each sample was precipitated with saturated ammonium oxalate. After standing for 24 hours, the samples were centrifuged for 5 minutes at 1200 r.p.m. (International Centrifuge, No. Y 4080). Most of the liquid was removed by suction, and the precipitates were washed three times with 2% ammonium hydroxide saturated with calcium oxalate. After each washing the samples were centrifuged for 5 minutes at 1200 r.p.m. and the wash fluid removed by suction. The precipitate was finally dissolved in 3 ml of hot 2N sulfuric acid and the resulting solution was transferred quantitatively to a 25 ml Erlenmeyer flask.

The oxalate was reduced with 2 ml of standard ceric sulfate (0.01 M). After 30 minutes, the excess ceric sulfate was titrated with standard ferrous ammonium sulfate (0.005 M), with ferroin as the indicator (Larson and Greenberg, '38).[3]

RESULTS

A first series of 17 experiments was done in which the total calcium content of each of 17 pairs of frog gastrocnemei was determined in order to assay the variability in muscle calcium content from frog to frog (table 1). The data show a great variation among frogs with respect to the wet weight and the dry weight, a variation that is not explained by the amount of water present. The amount of calcium present in the fibers also varies greatly. There is no apparent correlation between

[3] In the original paper by Larson and Greenberg there is an error in the statement of chemical equivalence. One milliliter of 0.01M ceric sulfate is equivalent to 0.2 mg of calcium (not 0.4 mg of calcium as there stated).

the amount of calcium and the weight of the fibers, either wet or dry. Therefore, the amount of calcium present has been given as mg/gm wet weight to permit comparison with other determinations.

Few systematic investigations of the calcium content of frog muscle appear in the literature. Burns ('33) reported

TABLE 1

The calcium content of 17 pairs of frog gastrocnemei

EXPERIMENT	WET WEIGHT OF FIBERS	DRY WEIGHT OF FIBERS	TOTAL CALCIUM	CALCIUM	CALCIUM
	gm	*gm*	*mg*	*mg/gm wet weight*	*mg/gm dry weight*
1	.8340	.0807	.225	.261	2.79
2	1.6430	.2201	.269	.164	1.22
3	.9780	.1165	.256	.262	2.20
4	.9707	.1096	.275	.283	2.51
5	.7495	.0896	.194	.260	2.16
6	1.6538	.2447	.313	.189	1.28
7	1.4713	.2181	.281	.191	1.91
8	1.1193	.1430	.281	.236	2.35
9	.8728	.1023	.256	.294	2.51
10	1.0900	.1453	.225	.206	1.55
11	.9583	.1196	.300	.313	2.51
12	.7270	.0772	.200	.275	2.59
13	1.3950	.2164	.288	.206	1.33
14	1.2433	.1778	.256	.206	1.44
15	1.2126	.1752	.238	.197	1.36
16	.7104	.0800	.225	.316	2.82
17	.8412	.0993	.269	.320	2.71

Mean, total calcium, mg/gm, wet weight = .245.
Standard error of the mean = .050.

the calcium content of separate right and left gastrocnemei of three frogs. For each frog, the combined right and left muscles give 12.0, 19.0, and 24.2 mg of calcium/100 gm of fresh tissue. Taubmann ('34) found variations of from 7.8 to 14.8 mg of calcium/100 gm of frog gastrocnemius muscle for 12 frogs. The range for milligrams of calcium/100 gm of wet weight found in table 1 is from 16.41 to 32.00, which seems to be in line with the few data in the literature. Possible ex-

planations for the variation in the calcium content might include such factors as age, degree of starvation, duration of captivity, and feeding periods before captivity.

Because of this wide variation among frogs, it seemed advisable to design the experiments so that this component of the variability could be segregated. It appeared that the method of analysis of variance was applicable for this purpose. In line with this design, the muscles of 4 frogs were pooled for each experiment and subsamples taken for each treatment.

The question can be raised as to whether the calcium in the initial soaking solution represents all the free calcium available; that is, whether there is more free calcium left in the treated muscle fibers that must be accounted for. If so, the method of ultrafiltration can be used to remove this residual free calcium. All remaining calcium would then represent the protein-bound calcium described by Greenberg and Gunther ('30). The sum of all calcium determinations, then, represents the total calcium content of the muscles and can therefore be used to test the validity of the sampling.

Table 2 represents the complete results for 11 experiments with all pertinent subdivisions. From this table are taken all of the following analyses. All figures are in terms of milligrams of calcium/grams of wet weight of fibers. From the table of means (table 2a), it can be seen that the means of the total calcium content of the muscle fibers are about twice as large as the means of total calcium given in table 1. However, the standard errors are about the same. It would seem, then, that although the mean value of the total calcium content of the muscle fibers depends upon the factors previously mentioned, nevertheless, the variability about the means seems to be consistent within the particular group.

Table 3 gives the analysis of variance for the total calcium measured in each experiment. This tests the validity of the sampling for each condition within each experiment. For treatments, $F = 2.03$, which is not significant for three and 30 degrees of freedom ($F_{.01} = 4.51$). The inference can be made that sampling was adequate within each experiment.

TABLE 2

Experimental data

(Calcium in mg/gm of wet weight of muscle)

NO.		SOLUTIONS			
		Ringer	2% ether	2% cocaine	0.4% chloroform
	Free Ca in soak	.124	.266	.233	.225
	Free Ca in fibers	.118	.077	.093	.086
1	Total free Ca	.242	.343	.326	.311
	Bound Ca in fibers	.219	.170	.116	.151
	Total Ca	.461	.513	.442	.462
	Free Ca in soak	.140	.190	.176	.176
	Free Ca in fibers	.096	.101	.107	.122
2	Total free Ca	.236	.291	.283	.298
	Bound Ca in fibers	.133	.222	.129	.151
	Total Ca	.369	.513	.412	.449
	Free Ca in soak	.058	.263	.093	.098
	Free Ca in fibers	.116	.105	.123	.137
3	Total free Ca	.174	.368	.216	.235
	Bound Ca in fibers	.256	.019	.149	.208
	Total Ca	.430	.387	.365	.443
	Free Ca in soak	.140	.251	.174	.272
	Free Ca in fibers	.202	.101	.074	.097
4	Total free Ca	.342	.352	.248	.369
	Bound Ca in fibers	.197	.100	.112	.121
	Total Ca	.539	.452	.360	.490
	Free Ca in soak	.097	.221	.223	.232
	Free Ca in fibers	.191	.176	.186	.166
5	Total free Ca	.288	.397	.409	.398
	Bound Ca in fibers	.088	.053	.069	.023
	Total Ca	.376	.450	.478	.421
	Free Ca in soak	.198	.236	.182	.201
	Free Ca in fibers	.063	.065	.079	.115
6	Total free Ca	.261	.301	.261	.316
	Bound Ca in fibers	.181	.095	.126	.078
	Total Ca	.442	.396	.387	.394
	Free Ca in soak	.117	.203	.198	.252
	Free Ca in fibers	.084	.082	.065	.058
7	Total free Ca	.201	.285	.263	.310
	Bound Ca in fibers	.197	.090	.053	.049
	Total Ca	.398	.375	.316	.359

TABLE 2 *(continued)*

NO.		SOLUTIONS			
		Ringer	2% ether	2% cocaine	0.4% chloroform
	Free Ca in soak	.141	.203	.180	.198
	Free Ca in fibers	.072	.105	.056	.086
8	Total free Ca	.213	.308	.236	.284
	Bound Ca in fibers	.108	.020	.114	.078
	Total Ca	.321	.328	.350	.362
	Free Ca in soak	.097	.151	.128	.143
	Free Ca in fibers	.082	.064	.073	.058
9	Total free Ca	.179	.215	.201	.201
	Bound Ca in fibers	.195	.198	.180	.152
	Total Ca	.374	.413	.381	.353
	Free Ca in soak	.083	.160	.116	.179
	Free Ca in fibers	.131	.091	.101	.094
10	Total free Ca	.214	.251	.217	.273
	Bound Ca in fibers	.304	.226	.233	.224
	Total Ca	.518	.477	.450	.497
	Free Ca in soak	.195	.422	.291	.278
	Free Ca in fibers	.070	.086	.076	.088
11	Total free Ca	.265	.508	.364	.366
	Bound Ca in fibers	.203	.095	.101	.080
	Total Ca	.468	.603	.468	.446

TABLE 2a

Table of means

	SOLUTIONS			
	Ringer	2% ether	2% cocaine	0.4% chloroform
Free Ca in soak	.126	.233	.181	.205
Free Ca in fibers	.111	.096	.094	.101
Total free Ca	.238	.329	.275	.306
Total calcium	.427	.446	.401	.425

Mean, total calcium mg/gm wet weight = .425.
Standard error of the mean = .063.

Table 4 gives the analysis of variance for the free calcium determined in the initial soaking solution. For treatments, $F = 16.47$, which is significant at the 1% level for three and 30 degrees of freedom ($F_{.01} = 4.51$). The inference can be made that a true difference exists in the amount of calcium liberated

by various treatments. From the three degrees of freedom for treatments can be segregated one degree of freedom with its corresponding sum of squares for the comparison of no anesthetic with the three anesthetics (Snedecor, '46). The corresponding F is 38.54 for one and 30 degrees of freedom ($F_{.01} = 7.56$). That is, there is a true difference in the amount of calcium released by the anesthetics used and the control Ringer solution. A further comparison can be made of the

TABLE 3

Analysis of variance of sampling

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	43	.171303	
Treatments	3	.011368	.003789
Experiments	10	.103880	.010388
Error	30	.056055	.001868

For treatments, $F = 2.03$ $F_{.01} = 4.51$.

TABLE 4

Analysis of variance of free calcium in soaking solution

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	43	.202303	
Treatments	3	.067873	.022624
Experiments	10	.093215	.009321
Error	30	.041215	.001374

For treatments, $F = 16.47$ $F_{.01} = 4.51$.

two fat solvent anesthetics with cocaine, for which F is equal to 7.63 for one and 30 degrees of freedom ($F_{.01} = 7.56$). That is, there is a true difference between the amount of calcium released by ether and chloroform on the one hand and the amount released by cocaine on the other.

Table 5 gives the analysis of variance for the free calcium determined in the ultrafiltrate. For treatments, $F = 1.34$, which is not significant at the 1% level for three and 30 degrees

of freedom ($F_{.01} = 4.51$). This indicates that for all practical purposes the analysis of the initial soaking solution was adequate for measuring the amount of calcium released.

Table 6 gives the analysis of variance for the total calcium released. For treatments, $F = 11.70$, which is significant for three and 30 degrees of freedom at the 1% level ($F_{.01} = 4.51$). In effect, the interpretations are those of table 4.

TABLE 5

Analysis of variance of residual free calcium in fibers

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	43	.057462	
Treatments	3	.002004	.000668
Experiments	10	.040490	.004049
Error	30	.014968	.000498

For treatments, $F = 1.34$ $F_{.01} = 4.51$.

TABLE 6

Analysis of variance of total free calcium

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	43	.216726	
Treatments	3	.051527	.017076
Experiments	10	.121156	.012116
Error	30	.044043	.001468

For treatments, $F = 11.70$ $F_{.01} = 4.51$.

DISCUSSION

The colloid chemical theory of stimulation and anesthesia (Heilbrunn, '43) suggests that protoplasmic response to a stimulus is dependent upon a clotting reaction which is initiated by a calcium release. In the case of stimulating agents such as mechanical impact, electrical shock, ultraviolet radiation, heat, and cold, calcium is released from the cortex of the cell, passes to the interior and initiates the clotting reaction. Mazia and Clark ('36), by observing the formation

of calcium oxalate crystals in the interior of Elodea cells, showed that calcium did leave the cortex under the influence of such stimulating agents as mentioned above. Recently, quantitative proof has been offered by Woodward (personal communication) who found that after the gastrocnemius muscle of the frog had been impregnated with radioactive calcium, Ca^{45}, washed thoroughly, and then stimulated electrically, a considerable amount of Ca^{45} could be detected in the Ringer solution surrounding the muscles.

Anesthetics also cause a release of calcium from the cortex, but in the interior of the cell the calcium is somehow prevented from being bound. Thus, the clotting reaction is inhibited and the cell does not respond to stimulation. The results reported in this paper indicate that calcium is released upon treatment with anesthetics.

Other experiments have been reported that support the view that calcium is released under the influence of anesthetics. In 1922, Burridge reported that if beating frog hearts were perfused with cocaine so that the beating ceased they could be revived by perfusion with calcium. He also found that perfusion with a solution of cocaine and calcium inhibited the effect of cocaine. From these experiments he was led to postulate that cocaine, in some way, deprived the tissues of calcium. Subsequent work by Simon and Szelöczey ('28) showed that after the nerves of dogs had been perfused with cocaine, calcium was released. Mazia and Clark ('36) showed that if ether was used in strong concentration it caused a release of calcium from the cortex of Elodea cells into the interior. Daugherty ('37) showed that alcohols and ether cause the cortical protoplasm of amebae to become more fluid, indicating a release of calcium into the interior of the cell.

The medical literature contains many papers on the calcium content of blood following or during the administration of anesthetics. Emerson ('28) reported an 18% rise in the calcium content of the blood of dogs that had been anesthetized with ether. Andrews, Petersen, and Klein ('30) also showed a rise in calcium in blood following ether anes-

thesia. In the same paper they reported a rise in the calcium content of the brain and liver. Since it is difficult to remove traces of blood from brain and liver, and since no mention is made of perfusion of these organs, the rise may have been a reflection of the increased blood calcium. Renzo ('29) reported a rising calcium content in blood following treatment of animals with ether, chloroform, chloral hydrate, and morphine. (For further similar results see also Messina, '32; Blitstein, '38; Fischer, '28; and In, '32.) These rises in the blood calcium may be interpreted as indicating a loss of calcium from the muscular and nervous tissues. In contrast to the results given in the 7 papers cited above, are those of Běcka ('33). Although he found a rise in the calcium in blood following the administration of chloroform and morphine, he found that other anesthetics such as hypnal (chloralhydrate-antipyrin), amylene hydrate (dimethylethyl carbinol), veronal (sodium diethyl barbiturate), urethane, and ether could cause either a rise or fall in the calcium content, while magnesium salts and avertin (tribromethanol) caused a fall in the calcium content.

The mechanism of the calcium release, its binding, and the prevention of its binding in the interior of the cell, still remain to be clarified. One theory suggests that the calcium is bound in the cell cortex in a lipoprotein complex. In an analysis of red cell membranes, Parpart and Dziemian ('40) found that a chemical constituent of the membrane was a lipoprotein. The fact that ether and chloroform cause a release of calcium may be explained by the ability of these anesthetics to dissolve the lipid part of the complex, splitting it into calcium, lipid and protein. However, since cocaine also causes a calcium release, and since the amount of free calcium is not as large as that found after treatment with ether and chloroform, it might be inferred that not all of the calcium in the cell is bound in a lipid complex. The rather constant amount of bound calcium that remains even after treatment with the fat solvent anesthetics also argues for the presence of calcium in some other type of compound within the cell.

The chemical nature of the lipoprotein bindings, as well as other types of calcium binding, and a physico-chemical study of the properties of such complexes, would clarify many problems concerning the role of calcium in the cell.

SUMMARY

1. The amount of calcium present in untreated fibers of frog gastrocnemius muscle was found to be extremely variable.

2. More calcium was found to be released from muscle fibers treated with experimental anesthetic solutions than from muscle fibers treated with control Ringer solutions.

3. More calcium was released by the fat solvent anesthetics, ether and chloroform, than by cocaine.

4. Most of the calcium released was found in the experimental and control soaking solutions. However, a small residual amount of free calcium was found trapped in the muscle fibers.

5. The results indicate that the production of anesthesia in muscle cells is associated with the release of an unusual amount of free calcium.

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LITERATURE CITED

ANDREWS, E., W. F. PETERSEN AND R. I. KLEIN 1930 Calcium changes in brain in ether anesthesia. Ann. Surg., *92:* 993.

BĚCKA, J. 1933 Veränderungen der pH-Zahl des Blutes und des Mineralstoffechsels während der Narkose. Arch. exp. Path. Pharmakol., *170:* 377.

BLITSTEIN, I. 1938 Modifications physico-chimiques du sang après les interventions chirurgicales sans divers anésthésiques; le calcium et le phosphore inorganiques. Rev. belge sci. med., *10:* 412.

BURNS, C. M. 1933 The calcium content of muscle. Biochem. J., *27:* 22.

BURRIDGE, W. 1922 Experiments with cocaine. Arch. int. de pharmacodyn. et de therap., *26:* 115.

DAUGHERTY, K. 1937 The action of anesthetics on amoeba protoplasm. Physiol. Zool., *10:* 473.

EMERSON, W. C. 1928 The effect of ether anesthesia and shock on calcium of blood. J. Lab. and Clin. Med., *14:* 195.

FISCHER, H. 1928 Die Rolle des Calcium beim Zustandekomen von Narkose. Ztschr. f. exp. Path. Pharm., *138:* 169.

GREENBERG, D. M., AND L. GUNTHER 1930 On the determination of diffusible and non-diffusible serum calcium. J. Biol. Chem., *85:* 491.

HEILBRUNN, L. V. 1943 *An Outline of General Physiology,* 2nd ed., Saunders, Philadelphia.

IN, K. 1932 Influence of Panaz genseng on the calcium and potassium concentration of rabbit serum and the effect of a few narcotics. J. Chosen Med. Assn., *22:* 142.

LARSON, C. E., AND D. M. GREENBERG 1938 The analysis of calcium in blood and other biological material by titration with ceric sulfate. J. Biol. Chem., *123:* 199.

MAZIA, D., AND J. M. CLARK 1936 Free calcium in the action of stimulating agents on Elodea cells. Biol. Bull., *71:* 306.

MESSINA, R. 1932 L'influenza delle sostanze farmaco-dinamiche sull' equilibrio calcio-potassio. Arch. di farmacol. sper., *54:* 214.

PARPART, A. K., AND A. J. DZIEMIAN 1940 The chemical properties of the red blood cell membrane. Cold Spring Harbor Symposium, *8:* 17.

RENZO, A. 1929 Studi dul magnesio. III. Influenza dei narcotici sulla magnesiemia. Boll. Soc. ital. Biol. sper., *4:* 579.

SIMON, A., AND J. SZELÖCZEY 1928 Untersuchungen uber Kalium und Calciumgehalt der peripheren Nervenfasen. Biochem. Z., *193:* 393.

SNEDECOR, G. W. 1946 *Statistical Methods.* Collegiate Press, Ames, Iowa.

TAUBMANN, G. 1934 Muskelkalk bei Ca-freier Durchstromung. Arch. f. exp. Path. Pharm., *174:* 476.

WINTERSTEIN, H. 1926 *Die Narkose,* 2nd ed., Julius Springer, Berlin.

A CHARACTERISTIC RESPONSE OF THE ISOLATED FROG SKIN POTENTIAL TO NEUROHYPOPHYSIAL PRINCIPLES AND ITS RELATION TO THE TRANSPORT OF SODIUM AND WATER[1]

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FOUR FIGURES

In previous papers (Ussing, '48, 49a) it has been demonstrated that there exists a very close correlation between the rate of sodium transfer across the isolated frog skin and the potential differences across the skin. Under a number of different conditions it has turned out that the higher the net uptake of Na^+, the more positive is the solution bathing the inside of the skin relative to that bathing the outside. It was concluded, therefore, that the potential difference (P.D.) across the skin is primarily the result of the active transport of sodium (Ussing, '48, '49a).

Some years ago it was found that a commercial preparation of the neurohypophysis having pressor and antidiuretic activity (Insipidin) produced in the axolotl an increased uptake of Na^+ and Cl^- through the skin (Jørgensen, Levi and Ussing, '46). If the relationship between the rate of Na^+ uptake and the skin potential holds it is to be expected that posterior pituitary hormones might produce an increase in the P.D. across the isolated frog skin when applied in the fluid bathing the skin. We have found no information concerning the effect

[1] An abstract has appeared in Federation Proceedings, *9:* 46, 1950.

[2] On leave of absence from the Department of Physiology, Stanford University School of Medicine, Stanford, California.

of these hormones on salt uptake through the skin of the frog. It is known, however, that posterior pituitary preparations have a definite effect in bringing about an increase in the rate of water uptake through the skin of the intact frog (cf. Heller, '45) as well as an increased renal tubular reabsorption of water in mammals.

Whole whale posterior pituitary, as well as commercial preparations of the pressor and oxytocic fractions of the ox pituitary, were investigated for their effects on the P.D. across the isolated frog skin. When it was found that these substances were effective in bringing about an increase in the P.D. further experiments were carried out in order to determine their effect upon the influx and outflux of Na^+ and on the uptake of water through the skin.

METHODS

All experiments were carried out in September and October using abdominal skin from the green frog (*Rana esculenta*). The frogs were taken from a large outdoor tank immediately before use. The experimental procedure was that described earlier (Ussing, '49a) with a few modifications. In the tracer experiments reported below the chamber holding the outside solution was reduced in depth, so that with the same exposed area (6.6 cm^2) the chamber contained only 3.3 ml. Moreover, narrower tubing was used for circulating the solution. The result was that the total volume of the outside solution could be kept below 8 ml, and the circulation system would work as long as the volume was above 5 ml.

The air used for circulating the solutions was saturated with water by passing it through two bubbling flasks. To still further minimize the water loss by evaporation, the outlets of the funnels, where the air leaves the circulation system, were closed with corks. The capillaries used for making contact with the calomel electrodes passed through a narrow slit in the corks.

The chambers used for the inside solutions were of the old type (Ussing, '49a) holding 13 ml. Usually at the start of the

experiments the inside fluid volume was 25 ml and the outside fluid volume 10 ml. Ringer solution containing 115 mM per liter of NaCl was always used on the inside, and in all but three experiments Ringer solution diluted tenfold, containing 11.5 mM NaCl per liter, was placed in the outer compartment. The pH of all solutions was 8.1 to 8.3. The effects of pH on the potential difference across the skin and on the sodium transport have been considered by Meyer and Bernfeld ('46) and Ussing ('49a).

The procedures used for handling and determining Na^{24} were those described previously (Ussing, '49a). All figures for radioactivity are corrected for background and for decay. In most cases the total number of impulses counted was above 10,000, thus giving a statistical mean error of less than 1%. All determinations were made on duplicate samples.

Sodium concentration was determined with the Beckman flame photometer. Duplicate determinations usually agreed to within 1%. Chloride was determined by a Volhard titration as modified in this laboratory (Schnorr, '34; cf. Rehberg, '26).

RESULTS

1. *Influence of neurohypophysial principles on the P.D. across the isolated frog skin*

Under the experimental conditions described above the inside solution was always positive relative to the outside. Although fluctuations in the P.D. occasionally occurred in fresh preparations, the P.D. usually became stable within one hour and remained remarkably constant for periods of 6 to 8 hours. After mounting a frog skin and allowing sufficient time for the P.D. to become stable, the preparations containing neurohypophysial principles were added to the inside solution. The P.D was then followed at frequent intervals for periods up to 6 hours after addition of the hormones. The results of all experiments are given in table 1. The initial P.D. is that measured immediately before addition of the hormones. The maximum P.D. is the highest P.D. attained

TABLE 1

Influence of neurohypophysial principles on the potential difference across the isolated frog skin

Inside solution = Frog Ringer; outside solution = 1/10 Frog Ringer except where indicated. Hormone preparations added to the inside solution. The inside solution was always positive relative to the outside.

NEUROHYPOPHYSIAL PREPARATION	DOSE ADDED TO 25 ML	INITIAL P.D.	MAXIMUM P.D.	INCREASE IN P.D. IN 1 HOUR
		mv	*mv*	*mv*
A. Whale posterior pituitary. Fresh suspension	0.05 mg	22	58	33
	0.05 mg	64	72	8
	0.05 mg	45	77	24
	0.05 mg	31	75	27
	0.05 mg	33	83	49
	0.05 mg	48	73	26
	0.05 mg	50	84	18
	0.05 mg	63	76	10
	0.05 mg	47	100	32
	0.05 mg	48	77	19
	0.05 mg	104	109	3
	0.05 mg	34	50	14
	0.05 mg	62	83	14
	0.05 mg	61	76	11
	0.05 mg	49	89	25
	0.05 mg	57	74	14
	0.05 mg	50	69	18
(Outside = Ringer)	0.05 mg	21 [1]	57	13 [1]
(Outside = 1/100 Ringer)	0.05 mg	8 [1]	8	0 [1]
(Moulting)	0.05 mg	27 [1]	34	7 [1]
B. Insipidin	4.0 I.U.	53	75	10
	1.0 I.U.	67	82	13
	0.1 I.U.	77	81	4
	0.1 I.U.	53	86	17
	0.04 I.U.	58 [1]	..	5 [1]
	0.04 I.U.	6 [1]	..	37 [1]
	0.02 I.U.	58 [1]	..	4 [1]
	0.02 I.U.	51 [1]	..	2 [1]
C. Pitupartin	2.0 I.U.	42	65	23
	2.0 I.U.	70	80	10
	2.0 I.U.	48	80	27
	2.0 I.U.	51	75	20
(Moulting)	2.0 I.U.	20 [1]	24	4 [1]
	0.2 I.U.	52	75	19
	0.2 I.U.	10	45	34
	0.1 I.U.	28	57	26
	0.1 I.U.	32	74	33
	0.1 I.U.	43	93	42
(Outside = 1/20 Ringer)	0.1 I.U.	55 [1]	72	17 [1]
	0.06 I.U.	50 [1]	..	8 [1]
	0.04 I.U.	62 [1]	..	4 [1]

[1] Not included in figure 2.

after the hormone addition without respect to the time of its occurrence. Since the experiments were of different duration, and since the course of the P.D. after hormone addition varied from animal to animal (see below), this figure is an approximation only. In some experiments, for which no figure for maximum P.D. is given in table 1, the addition of other substances after one hour prevented determination of the maximum.

(a) Unfractionated whale neurohypophysis. Whole posterior pituitary gland of the blue whale *(Sibbaldus musculus)* was used as a source of neurohypophysial principles in some experiments. This material can be considered to be free of anterior lobe contamination, since in the cetacean hypophysis the pars nervosa is enclosed in a capsule and the anterior and posterior lobes are separated anatomically (Wislocki and Geiling, '36; Valsø, '38).

The two lobes of the whale hypophysis had been previously separated upon removal and placed in acetone. The acetone was removed under reduced pressure and the dried material was powdered and suspended in distilled water a few minutes before use. Usually 0.1 ml of a suspension containing 0.05 mg of the whole posterior lobe powder was added to the 25 ml of Ringer solution contained in the inside compartment. The effect was a rapid increase in the P.D. after a brief lag period. The results of two representative experiments are shown in figure 1. The type of response produced by the various hormone preparations did not appear to differ, so that the description below applied equally well to all of them.

The lag period between addition of the hormone and the rise in P.D. varied from three to 10 minutes. This is longer than the time requires for mixing of dyes added to the inner compartment and probably represents, in part at least, the time required for diffusion of the hormone through the corium. The lag period appeared to be somewhat shorter with large doses of hormones than with small doses. Following the lag period a rapid rise in P.D. ocurred. In some experiments it increased at a rate of 3 mv per minute for

periods of one to 10 minutes. This rapid rise in P.D. was linear in many experiments when the P.D. was plotted as a function of log time. Following the rapid rise in P.D. the rate of increase was attained. This slow rate was maintained in some experiments for two to three hours and was of the order of 0.1 mv per minute (curve A, fig. 1). In other

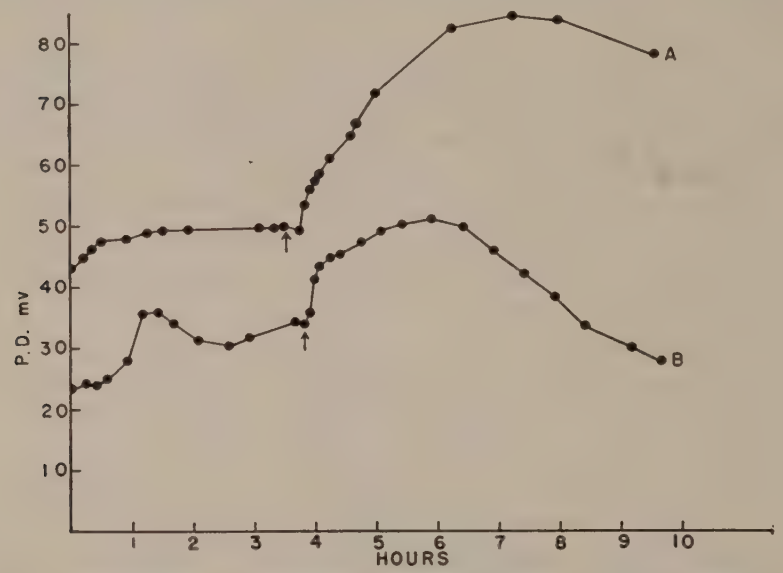

Fig. 1 Representative experiments showing the effect of the addition of a suspension of dried posterior pituitary of the blue whale on the potential difference across the isolated frog skin. Pituitary powder (0.05 mg) added to inside at arrows. Inside solution positive relative to outside.

experiments a fall in P.D. occurred several hours after addition of the hormone (curve B, fig. 1). This late fall in P.D. occurred chiefly in experiments lasting 10 hours, and since no substrate was added to the Ringer solution, it may represent exhaustion of endogenous substrate.

Aqueous solutions of the anterior lobe of the whale hypophysis in doses of 0.05 and 0.5 mg per 25 ml added to the inside chamber produced no increase in the P.D.

(b) Pressor and oxytocic fractions of the neurohypophysis. Commercial preparations of these fractions manufactured

by Alfred Benzon Co., Copenhagen, were used. Insipidin contained the antidiuretic and pressor (20 I.U. per millimeter) principles. Pitupartin contained the oxytocic principle (40 I.U. per millimeter). Neither of these preparations was pure and each was presumably contaminated with the other to the extent of about 5%. These preparations from freshly opened ampules were diluted 5 times with Ringer solution before addition to the inside solution. Since the inside pH influences both the P.D. and the sodium influx (Meyer and Bernfield, '46; Ussing, '49a) the possibility that these preparations altered the pH of the solution was examined. The addition of 2 I.U. Pitupartin to 25 ml of Ringer solution through which atmospheric air was bubbled produced a fall in pH of 0.6 units, but the pH returned to the initial level after 5 minutes. One-tenth this amount produced a fall in pH of 0.08 units, but the initial level was regained after two minutes. Since these decreases in pH were of such a transient nature it was considered that the effects obtained wth these hormones were not due to changes in pH.

Insipidin, in doses of 0.1 to 4.0 I.U. per millimeter produced an increase in P.D. across the frog skin which was similar to that obtained with whale posterior lobe powder. Since the rise in P.D. appears to depend upon the initial potential (see below) the minimum effective dose was not precisely determined. However, doses of 0.04 and 0.02 I.U. per 25 ml in 4 experiments produced less response than did higher doses when the level of initial P.D. was considered (table 1).

Pitupartin, in doses of 0.1 to 2.0 I.U. per 25 ml, also produced an increase in P.D. similar to that obtained with the other preparations. Doses of 0.06 and 0.04 I.U. appeared to be less effective (table 1).

These commercial preparations contained 0.5% chlorbutanol as a preservative. The addition of an aqueous 0.5% chlorbutanol solution in a volume up to 2.5 times the maximum volume of either Pitupartin or Insipidin used was without effect on the P.D.

After a rise in P.D. had been elicited by the addition of 0.1 I.U. per 25 ml or larger doses of either Pitupartin or Insipidin, the second addition of the same hormone was without marked effect. The addition of Pitupartin after a response had been obtained with Insipidin, or vice versa, in doses of 0.1 I.U. per 25 ml or larger, was likewise ineffective in producing a further increase in P.D.

Copper sulfate, 0.2 mg, added to the outside solution after a rise in P.D. had been obtained with Pitupartin or Insipidin (added to the inside) produced a further increase in potential similar to that observed after a simple addition of Cu^{++} to the outside solution bathing the skin of *R. temporaria* or *R. pipiens* (Ussing, '49b). The addition of 25 γ of adrenaline to the inside solution after previous addition of Pitupartin or Insipidin resulted in the characteristic rapid fall in P.D. described previously (Ussing, '49a). However, the secondary rise in P.D. following adrenaline was relatively much slower in the present experiments than in those reported earlier.

(c) Relationship between initial P.D. and the increase in P.D. produced by neurohypophysial principles. It is clear from the foregoing experiments that the intensity of the response to the various neurohypophysial principles largely depends upon the magnitude of the initial P.D. measured before addition of the hormone. Table 1 includes the figures for the increase in P.D. during one hour after addition of the hormones and the maximum P.D. attained during the experiments. The relationship between initial P.D. and the increase in P.D. during the first hours may be seen from figure 2. Selection of the one hour period as representative of the hormone action is arbitrary; a shorter period, however, would not always include the entire rapid rise phase. It may be seen from figure 2 that addition of the hormones when the initial P.D. was low produced greater effect than when the initial P.D. was high. There appears to be a tendency toward attainment of a maximum potential which cannot be increased further by hormone addition. The data are not sufficiently precise to warrant mathematical analysis. Figure 2 includes

30 experiments with whale posteror lobe, Insipidin and Pitupartin. Experiments in which the dose of Pitupartin or Insipidin was below 0.1 I.U. are not included in the figure. Two other experiments have been excluded. In one of these separation of the cornified epithelium was observed at the time of the experiment; it has been shown by Jørgensen ('49) that the permeability of the skin to water and salt is considerably increased during moulting. In this experiment whale posterior

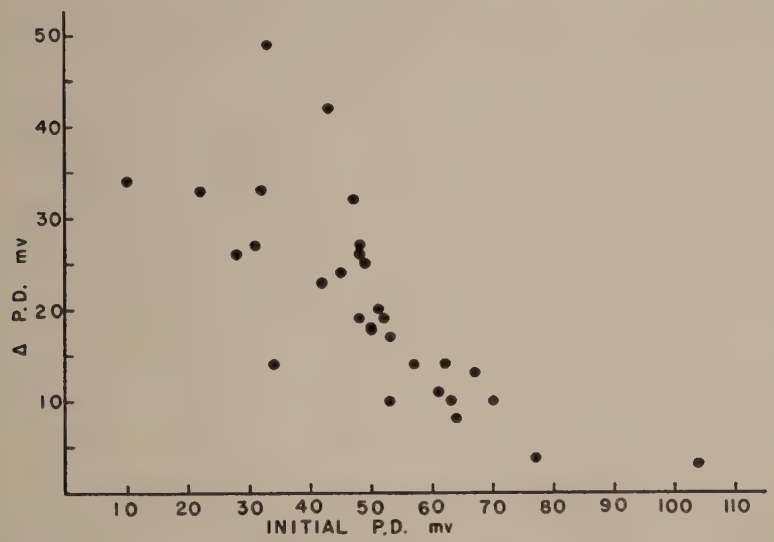

Fig. 2 Relationship between the initial potential difference across the frog skin and the increase in P.D. resulting from the addition of neurohypophysial principles to the solution bathing the inside of the skin. See table 1 for data.

lobe powder gave only a small increase in potential even though the initial P.D. was 27 mv (table 1). Determination of sodium outflux for this skin yielded values 5 to 10 times as high as those found in other skins. In the other animal actual separation of the cornified epithelium was not observed, but the response to Pitupartin was very small (table 1) and $CuSO_4$ addition to the outside solution failed to produce the characteristic rise in P.D.

(d) Effect of transfer of the inside solution to a second frog skin. In two experiments whale posterior lobe powder was added to the inside compartment and sufficient time was permitted for the characteristic rise in P.D. to take place. This fluid was then removed and added to the inner compartment of a second apparatus containing a fresh skin. The results are given in table 2. Since transfer of the hormone in this manner produced a response in the second skin it may be concluded that all of the hormone was not adsorbed on the first skin. Sufficient amounts remained in the circulating fluid to produce the rise in P.D. in the second skin.

TABLE 2

Transfer of Ringer solution containing whale neurohypophysial principles from one frog skin to another

Outside solution = 1/10 Ringer. Whole whale posterior lobe powder

SKIN NO. 1					VOL. TRANS.	SKIN NO. 2			
Dose	Vol.	Initial P.D.	Δ P.D.	Contact time		Inside vol.	Initial P.D.	Δ P.D.	Time
mg	*ml*	*mv*	*mv*	*min.*	*ml*	*ml*	*mv*	*mv*	*min.*
0.005	8	36	22.5	55	8	25	54	5	40
0.05	25	50	13	60	8	8	83	10	55

(e) Effect of outside Na^+ concentration. Only three experiments were carried out with outside NaCl concentrations other than 11.5 mM. The results are included in table 1. In the experiment with 1.1 mM NaCl outside, the potential was quite unstable and the result cannot be regarded as conclusive. It is clear, however, that the posterior lobe hormones are effective in producing an increase in P.D. with outside Na concentrations varying from 5.75 to 115 mM per liter. Subsequent experiments (Ussing and collaborators, unpublished) using skin from *R. temporaria*, *Bufo vulgaris* and *Bufo viridis* have established that in these species the effects of posterior pituitary hormones are as pronounced with Ringer on both sides of the skin as with 1/10 Ringer on the outside.

The relationship between outside Na concentration and the P.D. have been considered by Steinbach ('33), Meyer and Bernfeld ('46) and Ussing ('49a).

2. Influence of neurohypophysial principles on ion transport and water uptake

Although the pronounced effect of the posterior lobe hormone described above might conceivably be due to changes in behavior or in formation rate of a number of inorganic or organic ions in the skin, it seemed justified first to inquire whether the increase in potential difference after application of the hormone could be due to a change in behavior of the sodium in the system. Obviously an increase in potential could be due either to an increase in transport rate of Na^+ or to a decrease in the permeability to free Na^+ ions which, through back diffusion, tend to short the potential (cf. Ussing, '49a). It was planned to determine in each experiment: (1) the influx (or outflux) of Na^+, (2) the net change in outside Na^+ concentration, (3) the net change in outside Cl^- concentration (the inside solution was in all cases frog Ringer), (4) the changes in outside volume, and (5) the potential difference across the frog skin.

The recording of volume changes is particularly important in these experiments, because, as is well known (Heller, '45), posterior lobe preparations induce in amphibians an increased rate of water uptake through the skin. In periods this water uptake may, indeed, be so high that the outside salt concentration increases despite a relatively fast net salt transport inward. The estimation of changes in volume of the solutions was made indirectly from the changes in radioactivity of both solutions. The details are described below.

(a) Calculations. The calculation of the influx values presents no difficulties. We may take as an example the typical experiment shown in table 3. (The potential changes for the same experiment are shown in figure 1, curve B.) In the first 1½-hour period the Na^{24} activity per millimeter of the

inside solution increased by 745 counts per minute. In one hour, consequently, 477 × 24 counts went through the skin. Since in that period the outside radioactivity was 178,650 counts per minute per milliliter, and the Na^+ concentration

TABLE 3

Sample experiment

8:30	Frog killed. Abdominal skin excised and placed in vessel with Ringer solution.
8:50	Skin placed in apparatus. Outside compartment contained 10 ml 1/10 Ringer. Inside compartment contained 25 ml Ringer. Saturated atmospheric air used for mixing. Connected to potentiometer and P.D. read at intervals thereafter.
9:35	0.1 ml radioactive $Na^{24}Cl$, containing 53 mM NaCl by Cl titration added to outside compartment.
9:45	Samples from inside: 2 × 0.5 ml for Na^{24} counting. Samples from outside: 1 × 1.0 ml for Na analysis 1 × 0.181 ml for Na^{24} counting. 2 × 0.181 ml for Cl titration.
11:15	Samples from inside: 2 × 0.5 ml for Na^{24} counting.
12:45	Samples from inside: same as at 9:45 Samples from outside: same as at 9:45 0.1 ml of aqueous suspension of dry whale posterior lobe, containing 0.05 mg added to inside.
14:15	Samples from inside: 2 × 0.5 ml for Na^{24} counting.
15:45	Samples from inside: 2 × 0.5 ml for Na^{24} counting.
17:15	Samples from inside: 2 × 0.5 ml for Na^{24} counting. Samples from outside: 1 × 0.181 ml for Na^{24} counting.
18:45	Samples from inside: same at at 9:45 Samples from outside: same as at 9:45.

11.34 μM per milliliter, 0.726 μM have passed through in one hour. Since the exposed area was 6.6 cm^2, the influx was 0.11 μM per square centimeter × hour.

The water transfer in the first 3-hour period is found as follows: The Na^{24} activity per milliliter of outside solution at the start of the period was 179,000 counts per minute. At the end of that period the activity was 178,300 counts per

minute. Since the activity of the inside solution increased by 745 counts per minute per milliliter during the first 1½ hours, and by 639 counts per minute per milliliter during the second 1½ hours, $745 \times 24 + 639 \times 23$ counts per minute had passed into the inside solution during the 3-hour period. Designating the unknown volume at the end of the period by V, we have

$$V = \frac{(179{,}000 \times 8.56) - (32{,}577)}{178{,}300} = 8.42 \text{ ml.}$$

Since the original outside volume was 10.1 ml, and 1.54 ml were removed in sampling, then $8.56 - 8.42 = 0.14$ ml have disappeared; control experiments have shown the evaporation to be negligible, so therefore the water must have passed into the inside solution.

Evidently the activity per milliliter of the outside solution has to be determined with the highest accuracy possible. The samples used for determining the volume changes in some of the experiments were taken with a 100 µl Krogh syringe pipette which is accurate to at least 0.1 µl. These samples, due to their high activity, were left to decay in a protected place for several days before they could be counted. Simultaneously with one of these samples, however, samples of outside solution were taken which were diluted with Ringer solution so that they could be counted at once as were the inside samples. These latter outside samples were taken to give the true outside activity at a given time of the experiment. The outside activity at other times of the experiment were then calculated from the counts of the undiluted samples. The volume of the inside solution was sufficiently large as to make necessary corrections of volume due to water transfer. When we know the volume changes of the outside solution it is an easy matter to calculate the net uptake (or loss) of Na^+ and Cl^-. Since, however, the uncertainty of the volume determinations is about $\pm$ 100 µl, the net ion uptake is subject to an uncertainty which is at least equal to the amount of ion in question contained in 100 µl of solution, in the present case around one micromole. Since the net Na transport

is of the order of one micromole per hour $\times$ 6.6 cm^2, it is obvious that the net ion uptake can be determined with a fair degree of accuracy only if the experimental periods are of many hours duration. In the present experiments the apparent net ion uptakes are of limited value only, and the true rates of net uptake are found with far better accuracy

TABLE 4

Water uptake by frog skin

Calculated from radioactive Na measurements as described in text

Whale posterior lobe suspension added to inside solution. Outside compartment contained 1/10 Ringer. Inside compartment contained Ringer solution. Zero time is that of first sampling, 10 minutes after additon of Na^{24} (see table 3)

	H_2O uptake before hormone ml/cm^2/hr. 0–3 hours	H_2O uptake after addition of hormone ml/cm^2/hr.		
		3–9 hours	3–6 hours	6–9 hours
	0.005		0.018	
	— 0.010	0.012		
	0.009	0.017		
	0.007	0.012		
	0.007	0.016		
	0.020	0.019		
	0.008	0.014 [1]	0.016	0.012
	0.007	0.011 [1]	0.014	0.009
	0.012	0.020 [1]	0.031	0.009
	0.009	0.008 [1]	0.011	0.005
Mean	0.007	0.014	0.018	0.009

[1] Mean rate for 3–6 hours and 6–9 hours.

by comparing the rates of outflux with the rates of influx, even though the experiments were made on different animals.

In the outflux experiments the volume changes cannot be found as described above because the high activity is present in a large volume which is only changed by a few per cent by the water transfer. Here, therefore, the average water transfer found in the influx experiments is used for correcting the outside volume. This procedure is justified by the relative constancy of the volume changes found in the influx experiments.

(b) Results of water uptake experiments. Table 4 gives a comparison between the rate of water loss from the outside solution before and after addition of 0.05 mg of whale posterior lobe to the inside solution. During the 6-hour period after addition of the hormone the average disappearance

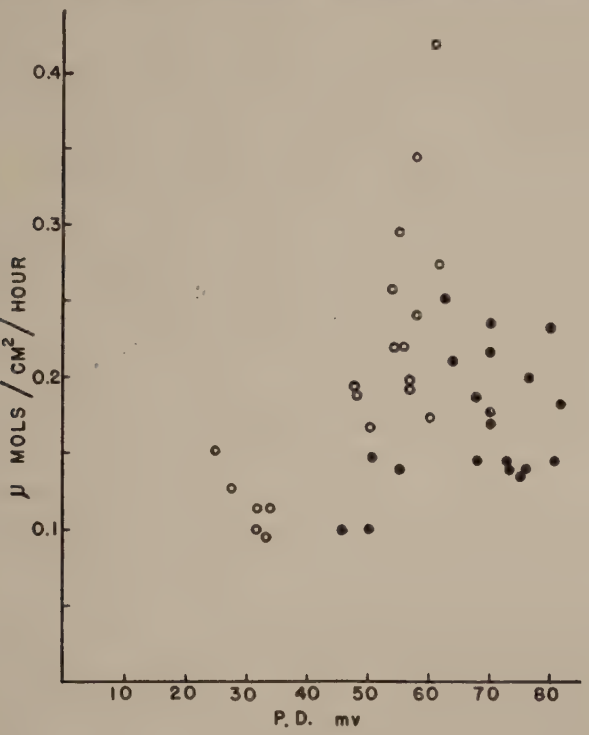

Fig. 3 Effect of whale posterior pituitary on the influx of Na^+ as a function of the potential difference across the frog skin. Each period of measurement of influx was 90 minutes. Open circles are before addition of the hormones; solid circles are after addition.

rate was 0.014 ml per square centimeter per hour compared to 0.007 ml per square centimeter per hour in the 3-hour control period immediately before addition. Statistical analysis by the method of paired differences indicates that such a difference would occur by chance in only 2% of the cases. In 4 experiments the water uptake was measured separately for

the period 0 to 3 hours and 3 to 6 hours after addition of the hormone. These results show that the effect of the hormone in increasing the rate of water uptake from the outside solution was more pronounced during the first 3 hours after addition than subsequently. From 3 to 6 hours after addition of the hormone the rate of water uptake declined, and at the same time the P.D. across the skin tended to fall in many experiments (for example, fig. 1, curve B).

This effect of neurohypophysial principles on water uptake corresponds as to sign and magnitude to what has been found in living frogs injected with posterior pituitary hormones (Jørgensen, '50).

(c) Results of ion transport experiments. The effect of the whale posterior lobe hormones on the influx of Na^+ is shown in figure 3. The influx is plotted against the average P.D. for each period. From each of 12 experiments 4 periods each 1½ hours in length have been included in the figure, namely two periods immediately before the hormone addition (open circles) and two periods following addition (solid circles). The figure seems to justify two conclusions: (1) the sodium influx increases with increasing P.D., and (2) after the addition of the hormone a higher potential is obtained for a given sodium influx.

Figure 4 shows a similar plot of the outflux values from 5 different experiments. It is seen that the outflux is lower at all potential values than was the influx. The outflux seems to increase somewhat with increasing P.D., but the slope is much less steep than is the slope for the influx. The net Na influx therefore increases with increasing P.D. There seems to be a tendency for the outflux values to be lower for a given P.D. than they were before addition of the hormone, but the material is too small to show this definitely.

Results of the determination of net sodium influx by *chemical* measurement of Na^+ concentration and calculation from the volume changes of the outside solution are, as mentioned above, subject to considerable uncertainty due to the limitations of the experimental methods. However, these

results also indicate an increased net uptake of Na^+ after addition of the hormone. The net Na^+ influx before addition of the hormone ranged from 0.010 to 0.180 μM per cm^2 per hour in 9 animals. In one other skin, however, it was 0.41 μM per square centimeter per hour. If this latter result is excluded from the calculations, the mean net Na^+ influx for the 3-hour control period before addition of the hormone was 0.086 μM per cm^2 per hour and for the 6-hour period after hormone addition it was 0.129 μM per cm^2 per hour.

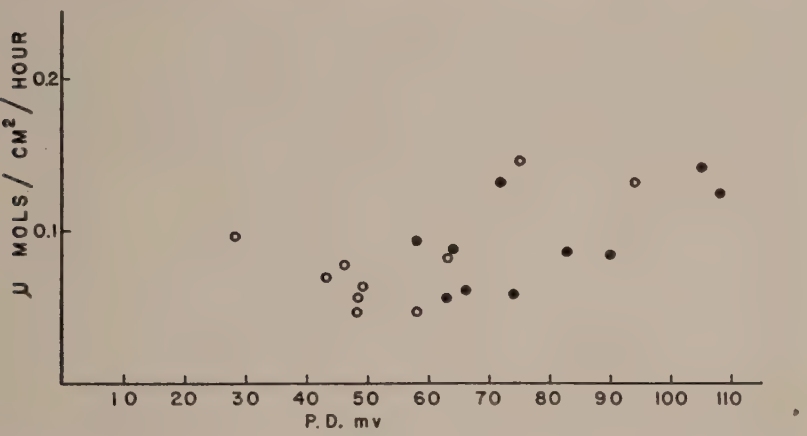

Fig. 4 Effect of whale posterior pituitary on the outflux of Na^+ as a function of the potential difference across the frog skin. Each period of measurement of outflux was 90 minutes. Open circles are before addition of the hormones; solid circles are after addition.

This difference in net Na^+ influx determined chemically before and after addition of the hormone was not significant by the "t" test ($P = 0.2$).

DISCUSSION

1. Neurohypophysial principles and potential

Of the many substances which influence the potential of isolated frog skin (for references see Dean and Gatty, '37 and Barnes, '39) the great majority decreases it. Of those few which produce an increase H_2O_2 (Marsh and Carlson, '41;

Carlson and Marsh, '43) and 2,4-dinitrophenol (Carlson, Martin and Krauel, '47) increases both the P.D. and the oxygen consumption, but over different concentration ranges. Eosin (Barnes, '39) increases the P.D., but only increases the oxygen consumption in some instances. Acetylcholine increases the electrical potential, but has little or no effect on the oxygen consumption (Barnes. '40). The addition of traces of Cu^{++} also increases the potential (Ussing, '49b), but comparable concentrations of this cation have no effect on oxygen consumption (Wennesland and Ussing, unpublished). In view of the possible relationship between potential and oxygen consumption (cf. Lund, '47) experiments were subsequently carried out to test the effect of posterior pituitary hormones on oxygen consumtion of frog skin (Fuhrman and Fuhrman, unpublished). At 20°C. Solution of Posterior Pituitary USP (Pituitrin, Parke-Davis) in a final concentration of 0.1 I.U. per milliliter increased the oxygen consumption of the skin of *R. pipiens* (18 determinations) and *R. catesbiana* (15 determinations) about 10%. However, chloretone, which was present as a preservative in the Pituitrin, when added to produce the same final concentration in the vessels, also increased the oxygen consumption about 10%. Posterior Pituitary Powder USP in final concentrations of 0.005 to 0.025 mg per milliliter did not significantly increase the oxygen consumption of *R. pipiens* skin. Probably it is not to be expected that an increase in the rate of oxygen consumption would be found, since the electrical energy output of frog skin constitutes such a small fraction of the total metabolic energy: 5 to 10% according to Francis ('33) or 1 to 2% according to Lund and Stapp ('47).

The course of increase in P.D. across the frog skin after addition of posterior pituitary hormones is similar to that reported by Barnes ('39) following the application of eosin. In both instances the effect is much more pronounced when the initial potential is low, and the final P.D. attained is not abnormal, but is of approximately the same magnitude as

the maximum potential observed in untreated skins. However, eosin increases the P.D. only when added to the outside of the skin; it produces a fall in P.D. when added to the inside only.

It would be of great interest to know which of the neurohypophysial principles is responsible for the characteristic effects on potential and sodium and water transport described here. Our data are not sufficiently precise to permit such a decision. It appears that Insipidin (containing the pressor and antidiuretic hormones) is slightly more effective than is Pitupartin (containing the oxytocic hormone, table 1). In the axolotl the injection of Insipidin brought about a net increase in Na^+ and Cl^- uptake through the skin, whereas Pitupartin injection produced a net Na^+ and Cl^- loss (Jørgensen, Levi and Ussing, '46). In experiments on water uptake through the skin of the living frog *(R. temporaria)* and toad *(Bufo bufo)*, Jørgensen ('50) has also found Insipidin to be somewhat more effective, at least in the latter animal. Heller ('45), however, has reviewed the literature concerning the effects of neurohypophysial principles on water balance in amphibia, and concludes that the hormone which produces the increased water uptake through the skin of living frogs ("Brunn Reaction") is associated with, but not necessarily identical with, the oxytocic fraction. Since the magnitude of the increase in potential produced by the neurohypophysial principles is dependent upon the initial P.D., and since pure preparations of the known hormones are not available, it would require large numbers of animals to demonstrate with certainty which fraction is responsible for the effect on the isolated frog skin.

2. *Neurohypophysial principles and sodium transport*

The influx and outflux of Na^+ remain very nearly the same after the addition of the posterior lobe preparations. Since, however, the work done by the skin upon the Na^+ is not only proportional to the net amount transported, but also to the

potential difference against which the transport is carried out, more effective Na^+ transport work is actually done after the addition of the posterior lobe extracts.

An increase in the net transport work may be brought about by at least three different mechanisms: (1) by actually increasing the transfer rate across one or more boundaries, (2) by reducing the back diffusion to free Na^+, and (3) by lowering the resistance to passive diffusion of Na^+ through some layer, thereby reducing the part of the total transport work dissipated in overcoming the resistance of the skin.

It is not possible from the material at hand to decide which of these possibilities is actually realized; subsequent work suggests that the third possibility is probably the correct one (Ussing and Zerahn, '51). It may be argued that the potential increase might have nothing at all to do with Na^+ transport. Recently, however, Ussing and Zerahn ('51; cf. Ussing, '50) have found that in totally short circuited frog skin the current comes solely from active Na^+ transport. It is therefore most likely that even in the non-short circuited skin the electric properties are closely correlated with the functioning of the Na^+ transport mechanism.

SUMMARY

1. An increase in the potential difference across the isolated skin of *R. esculenta* was produced by the addition to the solution bathing the inside of the skin of the following sources of neurohypophysial principles: unfractionated posterior pituitary of the blue whale, Insipidin (containing pressor and antidiuretic activity) and Pitupartin (containng oxytocic activity).

2. The increase in P.D. produced by these hormones was greater when the initial P.D. across the skin was low than when it was high. The maximum P.D. attained tended to be constant. Within wide limits the effect of these hormones on P.D. was nearly indipendent of the hormone dose.

3. Both with and without hormones the influx and outflux of sodium through the skin, measured with Na^{24}, tended to rise with increasing P.D.

4. The sodium influx was always higher than the sodium outflux, and the difference was greater the higher the P.D.

5. After addition of the hormones a higher P.D. was obtained at a given rate of sodium influx. Thus, after addition of the hormones, more of the work done by the skin appears to be available for formation of the P.D.

6. Net uptake of water from the outside solution increased after addition of the hormones.

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One of us (F.A.F.) would like to express his sincere thanks to the head of the laboratory, Professor P. Brandt Rehberg, and to his staff, for their many kindnesses shown to him and to his wife during the course of this work.

LITERATURE CITED

BARNES, T. C. 1939 The electrical potential of frog skin stained with fluorescent dyes. J. Cell. and Comp. Physiol., *14:* 83–94.

———— 1940 Modification of the electrical potential of frog skin by acetylcholine. Am. J. Physiol., *130:* 557–561.

CARLSON, L. D., AND G. MARSH 1943 The effect of hydrogen peroxide on the rate of oxygen consumption of frog skin. J. Cell. and Comp. Physiol., *22:* 99–114.

CARLSON, L. D., A. W. MARTIN AND K. K. KRAUEL 1947 Studies concerning the role of metabolism in steady EMF maintenance under the influence of DNP. Federation Proc., *6:* 87.

DEAN, R. B., AND O. GATTY 1937 The bioelectrical properties of frog skin. Trans. Farad. Soc., *33:* 1040–1046.

FRANCIS, W. L. 1937 Output of electrical energy by frog skin. Nature, *131:* 805.

HELLER, H. 1945 The effect of neurohypophysial extracts on the water balance of lower vertebrates. Biol. Revs., *20:* 147–158.

JØRGENSEN, C. B. 1949 Permeability of the amphibian skin. II. Effect of moulting of the skin of Anurans on the permeability to water and electrolytes. Acta Physiol. Scand., *18:* 171–180.

JØRGENSEN, C. B. 1950 The amphibian water economy with special regard to the effect of neurohypophyseal extracts. Danish Science Press Ltd., Copenhagen. Dissertation. Acta Physiol. Scand., *22:* Suppl. 78.

JØRGENSEN, C. B., H. LEVI AND H. H. USSING 1946 On the influence of neurohypophyseal principles on the sodium metabolism in the axolotl *(Amblystoma mexicanum).* Acta Physiol. Scand., *12:* 350–371.

LUND, E. J. 1947 Bioelectric fields and growth. Austin: University of Texas Press.

LUND, E. J., AND P. STAPP 1947 Biocoulometry. 1. Use of the iodine coulometer in the measuremen of bioelectrical energy and the efficiency of the bioelectrical process. In Lund, E. J.: Bioelectric fields and growth. Austin: University of Texas Press, 1947, p. 235–280.

MARSH, G., AND L. D. CARLSON 1941 The effect of hydrogen peroxide on the E.M.F. of frog skin. J. Cell. and Comp. Physiol., *18:* 317–328.

MEYER, K. H., AND P. BERNFELD 1946 The potentiometric analysis of membrane structure and its application to living animal membranes. J. Gen. Physiol., *29:* 353–378.

REHBERG, P. B. 1926 The determination of chlorine in blood and tissues by microtitration. Biochem. J., *20:* 483–485.

SCHNORR, E. 1934 A study of the cause of death in high intestinal obstruction. Dissertation, Copenhagen.

STEINBACH, H. B. 1933 The electrical potential difference across living frog skin. J. Cell. and Comp. Physiol., *3:* 1–27.

USSING, H. H. 1948 The use of tracers in the study of active ion transport across animal membranes. Cold Spring Harbor Symp. Quant. Biol., *13:* 193–200.

———— 1949a The active ion transport through the isolated frog skin in the light of tracer studies. Acta Physiol. Scand., *17:* 1–37.

———— 1949b The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin. Acta. Physiol. Scand., *19:* 43–56.

———— 1950 Active transport and diffusion of ions through living membranes. Introductory talk to general discussion. Abstracts of Communications, XVIII International Physiological Congress, Copenhagen, pp. 58–60.

USSING, H. H., AND K. ZERAHN 1951 To be published.

VALSØ, J. 1938 The hypophysis of the blue whale *(Balaenoptera musculus* L.). Macroscopical and microscopical anatomy and hormone content. Hvalrådets Skrifter, Det Norske Videnskaps-Akademi i Oslo, No. 16.

WISLOCKI, G. B., AND E. M. K. GEILING 1936 The anatomy of the hypophysis of whales. Anat. Rec., *66:* 17–41.

COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

STUDIES ON THE CONDUCTION OF SENSORY IMPULSES THROUGH THE DORSAL ROOT GANGLION IN THE FROG

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As a sensory impulse passes through the dorsal root ganglion there is a delay in addition to the conduction time. The delay of impulse is about 0.08, 0.14 and 0.23 msec in alpha, beta and gamma fibres respectively. The original observations of Erlanger, Bishop and Gasser ('26) are thus fully confirmed.

The delay, occurring at the T-shaped bifurcation point, is attributed to the limited quantity of energy provided by one single nerve impulse which is required here to excite two fibers. The delay is a consequence of the strength-duration re-

[1] I wish to thank Prof. J. C. Eccles for his many valuable advices and Drs. L. Brock, D. M. Easton and A. K. McIntyre for improving my manuscripts.

lationship of the stimulating current — in this case the eddy current produced by an impulse in the pre-bifurcation segment. The fact that one single nerve impulse can eventually excite two fibres is of course due to its comparatively large safety factor under normal conditions (cf. Rushton, '37 and Hodgkin, '37).

If a second impulse is set up in a sensory fibre early in the relatively refractory period, it will be conducted as far as the bifurcation point but no further. The reason for this blockage is simple. Although an impulse will propagate as soon as its safety factor is a little more than zero, it will not be able to excite two fibres at the same time unless its safety factor has recovered to a value of more than one. The blockage of impulses at the bifurcation point is shown to be responsible for the typical shape of the refractory curve as obtained first by Amberson and Downing ('29) and later by von Brücke, Early and Forbes ('41) with the dorsal root ganglion lying between the stimulating and recording electrodes.

The bifurcation point is a place where interaction between impulses of neighbouring fibres can easily take place. Thus, if a volley of impulses is set up in the tibial nerve during the relatively refractory period (T_2), the size of this volley of impulses as recorded in the dorsal root is easily affected by the presence of another volley passing along the peroneal fibres entering the same root (P_1). T_2 becomes larger, when P_1 precedes T_2, and smaller, when P_1 follows T_2, at very short time intervals in passing through the ganglion. These results are in general agreement with the observations of Katz and Schmitt ('40), Blair and Erlanger ('40) and Marrazzi and Lorente de Nó ('44).

By stimulating each of two branches of the N. cutaneus cruris lateralis with only one shock at various short time intervals and comparing the form of the composite potentials as recorded from many points along the nerve trunk, gan-

glion and dorsal root, another phase of interaction between nerve impulses in neighbouring fibres can be clearly demonstrated. When the interval between the two stimuli is less than 0.4 msec the impulses show predominantly synchronization. With longer intervals there appears to be also an effect of desynchronization. These effects should be detectable all along the nerve, but are more evident at the points of bifurcation. The negative results reported by von Brücke and Early ('41) were due to the fact that they recorded the potentials from one point alone and compared only the actual composite potential with the calculated sum of the two single volleys. With each volley consisting already of a great number of impulses, which also interact between themselves, such kind of comparison is no longer sensitive enough.

REFERENCES

AMBERSON, W. R., AND A. C. DOWNING 1929 J. Physiol., *68:* 1–18.
BLAIR, E. A., AND J. ERLANGER 1940 Am. J. Physiol., *131:* 483–493.
VON BRÜCKE, E. TH., M. EARLY AND A. FORBES 1941 J. Neurophysiol., *4:* 80–91.
VON BRÜCKE, E. TH., AND M. EARLY 1941 Ibid., *4:* 507–511.
ERLANGER, J., G. H. BISHOP AND H. S. GASSER 1926 Am. J. Physiol., *78:* 574–591.
HODGKIN, A. L. 1937 J. Physiol., *90:* 211–232.
KATZ, B., AND O. H. SCHMITT 1940 Ibid., *97:* 471–488.
MARRAZZI, A. S., AND LORENTE DE NÓ, R. 1944 J. Neurophysiol., *7:* 83–101.
RUSHTON, W. A. H. 1937 Proc. Roy. Soc., *B124:* 210–243.

THE TERMINAL ARBORIZATION OF NERVE FIBRES AS AN IMPORTANT FACTOR IN SYNAPTIC AND NEUROMUSCULAR TRANSMISSION

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In a preceding communication (Dun, '51) it is reported that the point of branching of sensory fibres inside the dorsal root ganglion is not only a site of impulse delay, but also

of ready interaction between impulses in neighbouring fibres. These observations are bound to be of some importance in interpreting the phenomena of synaptic and neuromuscular transmission, in view of the enormous number of terminal branchings which a nerve fibre shows in the vicinity of the cell it innervates. Following are some of the most salient points.

1. Owing to the delay at many points of branching the presynaptic or motor impulse probably comes to an end much later than was formerly supposed. Indeed, there is reason to believe that some of the impulses may even outlast the discharge of an impulse by the cell innervated (cf. the recent observation of Lorente de Nó, '50).

2. The detonator action and the two phases of facilitation obtained under conditions where no action of interneurons is possible (Eccles, '36; Lloyd, '46) may be explained as a result of synchronization and desynchronization of impulses in the presynaptic branches. The c. e. s. would then be regarded as the direct excitatory effect of the presynaptic impulses. This conception agrees well with the experimental results of Bremer ('42).

3. Both peripheral and central inhibition (direct as well as indirect) may now be explained on a common basis, i.e., the interaction between impulses at the points of branching. The concept that an impulse in the small nerve branches can be blocked by a neighbouring one even under normal conditions, is based on the supposition that the thinner the fibre the smaller is the safety factor of its impulses. If there are specific inhibitory fibres, these fibres must, according to this scheme, be thinner and with fewer branches than the excitatory fibres at the synaptic or neuromuscular junctional region. Thus in the picture of Lorente de Nó ('38, fig. 3) we may regard the presynaptic fibril 18 as inhibitory and 12 as excitatory in nature. For peripheral inhibitory fibre see van Haareveld ('39).

4. From the hypothesis mentioned above it seems likely that a presynaptic or motor impulse may not normally propa-

gate to the very end of the final branches of the nerve. In that case the transmission would take place between the cell innervated and the arborized fibres but not under their terminations, a possibility recognized long ago by Eccles ('36). Adopting the general idea of Eccles' ('46) recent theory but assuming that the scattered terminations act as "source" only, a large area of neural or muscular membrane underneath the branches can be easily visualized as subjected to the action of a homogeneous depolarizing field. This scheme will meet the physical considerations of Schoepfle ('47). And synaptic and ephaptic transmission (Arvanitaki, '42) would then seem very similar in nature indeed, with perhaps the only difference that in synaptic transmission the A_1 effect (cf. Eccles, '46) is more effectively reduced, while the C_2 effect greatly enhanced, by the terminal arborization.

5. Although each impulse in the terminal branches of an axon is all-or-nothing in character, yet the mutual interaction between impulses in different fibres leads to wide variation in the end result upon the innervated cell or cells resulting from the arrival of a single impulse in the afferent or motor axon. These variations will be in duration as well as in magnitude, in the time of beginning and in the exact location of the excitatory effect. The fixed histological distribution of the terminal branches provides very likely only a maximal topographical limit for the excitatory action.

REFERENCES

ARVANITAKI, A. 1942 J. Neurophysiol., *5:* 89–108.
BREMER, F., ET AL. 1942 Arch. intern. Physiol., *52:* 1–56.
DUN, F. T. 1951 J. Cell. and Comp. Physiol., 38: 131.
ECCLES, J. C. 1936 Ergeb. Physiol., *38:* 339–444.
———— 1946 Ann. N. Y. Acad. Sci., *47:* 429–455.
VAN HAAREVELD, A. 1939 J. Comp. Neur., *70:* 267–284.
LLOYD, D. P. C. 1946 J. Neurophysiol., *9:* 421–438.
LORENTE DE NÓ, R. 1938 Ibid., *1:* 195–206.
———— 1950 Abst. XVIII. Intern. Physiol. Cong., p. 45.
SCHOEPFLE, G. M. 1947 J. Neurophysiol., *10:* 339–347.

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CONTENTS

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